Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

AETE CONTRIBUTIONS (SBTE) – PROCEEDINGS 2024

From the AETE President

Dear friends and colleagues

It is my great pleasure to welcome you to the Annual Scientific Meeting of AETE in the beautiful city of Brescia, Italy as we celebrate the 40th anniversary of our association. The first meeting of AETE was held back in 1984, with Pierre Mauléon as the very first President of our society; Jeff Mahon, Vice President and Treasurer; and Jean-Claude Plat, the first Secretary. Since these early times AETE has grown, flourished and evolved to what it is today – a vibrant and friendly society that fosters connections between its members to interact and exchange knowledge across Europe and worldwide with our partner societies IETS and SBTE, as well as numerous national societies.

This milestone anniversary is a perfect occasion for reflection and celebration. We will revisit the decades via the path that has led our society and the ART industry to our current achievements and where we are heading. Our outstanding speakers from Europe, Brazil and the US will share their diverse perspectives, guiding us through this voyage. In addition to these invited talks, the program will include two practical workshops, the student competition and oral presentations of selected abstracts. With four poster sessions, there will be ample opportunity to discuss with the presenters about their latest research. We are also thrilled to honor Patrick Lonergan with the Pioneer Award of 2024 for his distinguished career.

Kicking off the event, the Local Organizing Committee, chaired by Pierluigi Guarneri, has prepared a Preconference Workshop. The workshop, focused on bovine species including the buffalo, will delve into the origins of fertility, starting from the in utero period to the management of young animals, collection of oocytes, and finally grading of oocytes and IVP embryos. This event will take place at the Agricultural Technical Institute barn facilities a short distance from our conference venue.

The scientific sessions will be hosted at The Centro Pastorale Paolo VI. The LOC has prepared a delightful social program starting with a Welcome Reception taking place in the beautiful garden of Paolo VI. Our Gala Dinner will be an evening under the arches of the Vita restaurant accompanied by jazz music and followed by dancing. Since we are in the “home” of pizza, what better way to bid farewell than with a party at a local pizzeria, enjoying a buffet of delicious pizzas and Italian specialties. After the main event, the conference will conclude with an exciting post-conference tour to Avantea in Cremona.

I extend my heartfelt thanks to everyone involved in organizing this special anniversary meeting. The dedication of the LOC and the board of AETE have been instrumental in making this event a success. I am deeply grateful to our loyal sponsors and new supporters for their generous contributions. A special thank you to our invited speakers as well as all researchers sharing their latest work and all members and friends of our society for making this event possible.

Let’s make this 40th anniversary meeting a memorable and inspiring celebration!

Marja Mikkola
President of AETE

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AI / TAI / FTET

Mapping of ADAMs and ADAMTs expression in response to mating, or seminal plasma infusion in periovulatory sows

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Boar semen (sperm and seminal plasma (SP)) modulates gene expression, from immune to general metabolism processes, in the preovulatory female genital tract. Some genes can be affected for later events, where the extracellular matrix (ECM) is modified, as during embryo implantation. Desintegrin metalloproteinase (ADAMs), and ADAM with thrombospondin motifs (ADAMTS) are a single-pass transmembrane zinc-dependent family of proteinases, whose biological function mainly depends on protein-protein interactions, becoming key-remodeling enzymes of the ECM. This study aimed to assess if sperm and/or SP triggers changes in *ADAM* and *ADAMTS* mRNA expression in the pig periovulatory genital tract. Mucosal samples (cervix (Cvx), uterus (distal (DistUt) and proximal (ProxUt)), utero-tubal junction (UTJ), isthmus (Isth), ampulla (Amp) and infundibulum (Inf)) were surgically removed from sows 24 h after natural mating (NM, n=4, to a single Swedish Landrace male each), and, with pooled seminal plasma collected from the same males, cervical infusion with 10 mL of sperm-peak fraction (P1-AI, n=4) or of sperm-free SP from the sperm-peak fraction (SP-P1, n=4) or the whole ejaculate (SP-Total, n=4). Infusions with the protein-free extender Beltsville Thawing Solution (BTS) were used as the control group (n=4). RNA was isolated following a TRIzol-based modified protocol and analyzed for global transcripts using specific microarrays (PORGENE 1.0 ST GeneChip® array, Affymetrix). Normalization of the data (Robust Multiarray Average) and mRNA differential expression (ANOVA: -1> fold change >1, $p<0.05$) were analyzed with the Transcriptome Analysis Console, and molecular processes were identified by PANTHER. Results, although showing a plethora of differential mRNA expression, clearly marked NM and SP-infusions as downregulators of *ADAMS*. NM induced the downregulation of *ADAMTS4* in Cvx, DistUt, and ProxUt, and of *ADAM12* (a gene closely related to the expression of estrogens and progesterone) in DistUt, ProxUt and UTJ. *ADAMTS9* mRNA downregulation was only found in sperm-containing treatments (NM and P1-AI), in ProxUt. ADAMTS9 is an angiogenesis inhibitor, so perhaps our results are related to the preparation of the endometrium for later reproductive events. In addition, SP-infusions also triggered a notable downregulation of *ADAMs*, but only in the distal segments of the genital tract (Cvx to ProxUt). An isotype (*ADAMTSL4*) was upregulated in Isth, Amp, and Inf and *ADAMTS16* was upregulated in ProxUt after mating, P1-AI and SP-AI, *which* enhances the expression of MMP9 (a tissue inhibitor), degrades ECM, and promote the invasion of trophoblasts. In conclusion, the global downregulation pattern of *ADAMs*and *ADAMTSs* in response to NM might be related to the periovulatory phase, where the ECM remodeling activity is lower, or to SP in the distal parts of the genital tract, which might be related to signaling cascade in the female genital tract. Supported by The Swedish Research Council FORMAS (2017-00946 and 2019-00946), Stockholm, Sweden, and RYC2020-028615-I, RyC2022-036771-I, PID2022-136561OB-I00, and CNS2023-144564, funded by MCIN/AEI/10.13039/501100011033 (Spain) and FEDER funds (EU).

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AI / TAI / FTET

Factors affecting recipient rejection rate in embryo transfer programs in lactating dairy cows

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Efficiency in dairy cattle farming is essential for maximizing profitability. Incorporating timed embryo transfer (TET) programs into breeding strategies can enhance efficiency by transferring purebred beef embryos in cows not required to generate replacement females, thereby increasing the beef value of surplus calves. Additionally, TET has the potential to improve fertility during warmer seasons and avoid undesired twin pregnancies. Yet, the efficiency of TET programs in high-producing lactating dairy cows must be evaluated based on the rejection rate (RR), pregnancy per ET, embryo loss rate, and cost-benefit ratio achieved. Non-eligible dams for ET among the synchronized cows, as defined by RR, may be influenced by factors such as season, parity, days in milk (DIM), body condition score (BCS), and synchronization protocol. Thus, we aimed to evaluate the effect of such factors in RR in high-producing lactating Holstein cows under confinement systems. The estrus of 331 cows from two farms in southern Spain were synchronized (Double Ovsynch in farm 1 or G6G in farm 2 using D-cloroprostenol [Veteglan, Calier, Spain] and Buserelin [Veterelin, Calier, Spain]) from July 2023 to April 2024. On d 7 after presumptive estrus, reproductive tracts were examined by transrectal ultrasound to assess corpus luteum (CL) status and number (multiple ovulation rate [MOV]: proportion of cows with >1 CL among all ovulated cows), presence or absence of cyst structures, and uterine health status. On the same day, BCS, DIM (89.8 ± 17.8; mean ± SD) and parity (2.2 ± 1.3) were recorded. To calculate RR, the number of unsuitable cows for TET was determined on the basis of having no CL present, abnormal ovarian structures, or small CL (<15 mm). Statistical analyses were conducted using a generalized linear mixed model (GLIMMIX, SAS) to assess the effects of independent variables on the binary response variables (RR and MOV). Fixed effects included farm, season (warmer and cooler), parity (1st, 2nd, and ≥3rd lactation), DIM (<90 d, and ≥90 d), BCS (≤2.50, 2.75, 3 and >3), and synchronization protocol. All effects and two-way interaction were tested and manually removed by backward elimination if non-significant (P>0.05). Overall, RR was 7.2% and was not affected by season, parity, DIM, BCS, or synchronization protocol. As a whole MOV was 13.7%, and it was higher in the warmer (27.1%) than in the cooler season (8.8%; P<0.05). Moreover, ≥3rd lactation cows had a higher MOV (20.8%) compared to 1st and 2nd lactation cows (10.4% and 10.3%, respectively; P<0.05). In conclusion, MOV was higher during the warmer season and in ≥3rd lactation cows, regardless the season, increasing the risk of twin pregnancy with artificial insemination. In such circumstances, TET could potentially be applied widely, as RR is not affected by these factors. Future studies should assess pregnancy per ET, embryo losses, and cost-benefit ratio in these conditions to further confirm the efficiency of this strategy. Funds: MCIN/AEI/10.13039/501100011033 and European Union Next GenerationEU/PRTR (RYC2021-033574-I and TED2021-129764B-I00).

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AI / TAI / FTET

Long term study of the blood plasma biochemical profile of cattle born by assisted reproductive technologies

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Assisted reproductive technology (ART) is key in cattle breeding as it enables selective mating and improves both reproductive efficiency and genetic improvement. However, there is still limited information on the long-term effects of these methods on the health and development of the offspring. This study aims to investigate the biochemical profile of cattle born through ART, from a previous study (Lopes *et al.*, 2020), over an extended period to understand how these techniques may affect their health and metabolism.

Male and female (not pregnant or lactating) cattle born after artificial insemination (AI, N= 7) were compared to those derived from *in vitro* produced embryos (IVP) using a standard protocol (C-IVP, N= 7), or using oviductal and uterine fluids in the process (RF-IVP, N= 4). Males and females were kept in two diferent open pens, but fed and managed under identical conditions their whole lives. Animals were studied every six months, from 1.5 years old until ≥4 years old, since their first year of life was evaluated by Lopes et al. (2022). Tail vein blood plasma was obtained by centrifugation (1000 G, 10 min) in lithium heparin tubes and stored at 80°C. The biochemical profile included total protein (TP), albumin (ALB), globulin (GLOB), creatinine (CREA), urea (URE), glucose (GLUC), cholesterol (CHOL), triglycerides (TRIG), amylase (AMIL), lipase (LIP), creatinine kinase (CK), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TB). After serial dilutions, inter- and intraassay precision of the methods were lineal and below 15%. Data normally distributed was analysed using ANOVA and Tukey's test; data not normally distributed was analysed using the Kruskal Wallis and Games Howell's test (statistical significance $p<0.05$). The data is shown below as means by group over the years.

The AI group had lower CREA and CHOL levels (1.43±0.05 mg/dL and 109.97±5.27 mg/dL, respectively) than C-IVP (1.75±0.07 mg/dL and 126.57±7.53 mg/dL, respectively) and RF-IVP (1.86±0.08 mg/dL and 140.01±7.19 mg/dL, respectively). The GGT levels were lower in RFIVP animals (13.00±1.30 UI/L) than both the C-IVP (17.55±1.10 UI/L) and AI groups (16.05±1.23 UI/L). Moreover, the levels of ALT were significantly lower in C-IVP (28.60±1.72 UI/L) than in the AI (34.35±1.81 UI/L) and RF-IVP groups (33.40±2.24 UI/L).

Depending on the age of the animal at the time of sample collection, significant differences were observed for most parameters. The parameters TP, GLOB, CREA, URE, AMIL, AST an increased in their concentration was observed as the animals aged, whilst ALB, GLUC, ALP decreased with age. However, all values can be considered within physiological ranges despite the significant variations.

To conclude, the study showed differences in some biochemical parameters amongst the ART groups and with aging. However, despite the diferences all values were within physiological ranges. These findings are key to study the potential implications of this diferences on the animal's health.

This study is part of project PID2020-113366RB-I00 funded by MCIN/AEI/10.13039/501100011033/ and "FEDER Una manera de hacer Europa".

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AI / TAI / FTET

Growth parameters and their hormonal regulation in pigs are differently affected by sex and embryo origin

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The global pig embryo trade has yet to emerge but replacing live animal transport with *in vitro* produced (IVP) embryos is a future objective. Understanding the potential long-term effects of *in vitro* protocols, especially in commercial traits like growth rates, is crucial. Our study aimed to compare growth parameters (body weight and length) and growth-related hormones (IGF-1 and T4) between IVP and artificially inseminated (AI) animals. Three experimental groups were defined, including 68 crossbred (Landrace x Large White) pigs that were allocated from a colony of animals produced in a previous study. IVP animals produced using BSA (C-IVP; 8 males, 16 females) or reproductive fluids (RF-IVP; 10 males, 7 females) as supplement for *in vitro* culture, were compared to AI animals (AI; 9 males, 18 females). Paternally, all animals were sired by the same boar, while maternally, IVP animals were originated from ovaries obtained at a local abattoir. Growth was measured at birth, 3, 9, 15, every 15 days until 180, 365, 700, 900, 1100, 1250, 1450, 1650 and 1800 days of age. Blood was collected at 90, 180, 365, 700 and 900 days of age. Plasma hormone concentrations were determined using a solid phase, enzyme-labeled competitive chemiluminescent immunoassay. Data were analyzed using a linear mixed-effects model, with group, sex and age as fixed effects. Benjamini-Hochberg procedure was used for p-value correction and differences were considered significant when $P< 0.05$. Weight exhibited an exponential increase up to 6 months of age followed by a linear rise from 12 months of age. Levels of T4 decreased with age, and IGF-1 levels also decreased with age, but only in females and C-IVP males. Males were longer and heavier than females throughout the study. Additionally, males exhibited higher IGF-1 levels than females, while the opposite was observed for T4. Only in males, T4 levels negatively correlated with weight, and no correlation was observed between T4 and body length. IGF-1 positively correlated with weight and length until 6 months of age. IVP animals were significantly heavier (≤d180: 10.6 ± 1.1 kg; ≥365: 35.9 ± 5.4 kg) and longer (≤d180: 8.5 ± 0.8 cm; ≥365: 10 ± 1.9 cm) than those originated from AI throughout the study. In addition, C-IVP animals were heavier (4.3 ± 1.5 kg) and longer (3.2 ± 1.1 cm) than RF-IVP but only up to 6 months of age. Finally, in males, T4 levels of AI were higher than those of their IVP counterparts (1 ± 0.2 µg/dL) and IGF-1 levels of RF-IVP were higher than those of AI (36.1 ± 11.5 ng/mL).

In conclusion, the growth and growth-related hormones of males and females exhibited differences throughout the study. Specifically, males exhibited higher levels of IGF-1, while females exhibited higher levels of T4. While statistical differences were identified between experimental groups, further studies with larger sample sizes are required to determine the clinical significance of these findings, if any.

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AI / TAI / FTET

Blood plasma biochemical parameters in fully-grown pigs derived from assisted reproductive technologies

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Emerging evidence indicates a long-lasting effect of assisted reproductive technologies (ART) on molecular physiology and metabolic function. Previous studies have reported variations in the metabolic profiles in piglets and growing pigs born from artificial insemination (AI) and born after transfer of in vitro produced embryo (IVP) (París-Oller et al., Res Vet Sci,142:43, 2021) but it is unknown whether these differences persist into adulthood. The aim of this work was to study baseline values of plasma biochemical parameters in ART-derived pigs and to gain insight into the evolution of metabolic profile across their life [young age (45 days), adulthood (365 days), and old age (1250 days)]. Pigs born through AI and IVP [n= 16 and 29 (45 days), 13 and 21 (365 days), and 9 and 13 (1250 days), respectively], produced in a previous study (París-Oller et al., J Anim Sci Biotechnol 12:32, 2021), were kept under same housing, managing and feeding conditions. Plasma was obtained through the centrifugation (1200 *g*, 20 min, 4 ºC, Eppendorf 5810 R) of blood collected in lithium heparin tubes and stored (-80ºC) to determine the biochemical parameters total protein (TP), albumin (ALB), globulin (GLOB), creatinine (CREA), urea (URE), glucose (GLUC), cholesterol (CHOL), triglycerides (TRIG), amylase (AMIL), lipase (LIP), creatinine kinase (CK), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (TB) using an automated clinical chemistry analyzer (Olympus AU400, Japan). Inter- and intraassay coefficient of variation were below 15%. Data were analyzed by a mixed ANOVA and Student's t-test for multiple comparisons. In the absence of homoscedasticity, a Student's t-test with Welch's correction was used, and in case of non-normality the Wilcoxon rank-sum test was performed. A P value < 0.05 was considered significant. The analyses of the metabolites showed higher CREA in old-IVP than old-AI (2.32±0.07 vs. 1.925±0.07 mg/dL); higher GLUC in young-IVP than young-AI (107.77±6.10 vs. 83.77±5.41 mg/dl); lower LIP in young-IVP than young-AI (16.74±1.29 vs. 22.91±2.11 IU/L); and lower AMIL, GGT and AST in IVP than AI at all ages. The other metabolites were similar between AI and IVP animals but increased (PT, ALB, GLOB, URE, TBIL), decreased (CHOL, ALP, ALT) or fluctuated up and down (TRIG) with age. The enzyme CK, related to muscle integrity, was the only parameter that was not affected by the group or age of pigs. Reference values for plasma biochemical values provide valuable information for investigators and will help in valid interpretation for health status and for those who use IVP pigs as a research model. In conclusion, these physiological data are useful for veterinarians and livestock producers and show slight persisting differences in some metabolites in pigs naturally and artificially conceived during life although the clinical relevance of such differences is unnoticeable.

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AI / TAI / FTET

Seasonal fertility variation in dairy cows in two farms in the Mediterranean area

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The ongoing climate changes remarkably affect the distribution of rainfalls and seasonal maximum/minimum temperatures, negatively influencing global agricultural activities. In this context, reproductive performance in dairy cows may decrease. The aim of this retrospective, observational study was to investigate the seasonal fertility variation in Holstein Friesian dairy cows of two commercial farms (F1, F2) located in Sardinia (Italy). A total of 4722 artificial inseminations (AI) in 800 lactating dairy cows (F1 n=300; F2 n=500) were examined over 3 years (2021-23). The monthly conception rate (CR) was calculated as the percentage of inseminated cows diagnosed as pregnant via trans-rectal ultrasound approximately 30 d post-AI. The number of services per conception (S/C) was calculated as the number of AIs needed to conceive per cow. Data were analyzed with R (RStudio Team 2023), by a simple linear regression model with fixed effects in which the two response variables were CR and S/C, and the predictor variables were farm, month, year and their interaction. Average (±SEM) monthly CRs in 2021-22-23 were 58±21%, 60±21% and 43±12% in F1 and 44±15%, 39±8%, and 46±12% in F2, respectively. The mean monthly CR was higher in F1 vs F2 (P<0.01). CRs did not vary within farms among years but were different among months (P<0.05) and farms (P<0.01), while there was no significant interaction between month and farm. Maximum CRs were achieved in winter/autumn (Dec, Jan, Feb, Sep), while minimum CRs were recorded in spring/summer (Apr, Jul, Aug) in both farms. The mean difference between the highest and lowest CR of the two farms in the 3 years was similar (F1=48.7% and F2=40.3% percent points difference). The yearly mean S/C were 2.06±0.36, 2.24±0.52, 2.45±0.34 in F1 and 2.58±0.34, 2.90±0.64, 3±0.52 in F2 in 2021-22-23, respectively. The highest monthly S/C during the three years were achieved during spring/summer (Apr, Jun, Jul) in F1, while the lowest S/C occurred in spring and autumn (Apr, Sep, Oct) in F2. As observed for the CR, the difference between highest and lowest S/C was similar between farms (F1=1.29; F2=1.53). The mean S/C was similar among months, while the variance was significant among years (P<0.02) and farms (P<0.01), but there was no significant interaction between year and farm. In agreement with previous reports, evidence indicates that reproductive performance in dairy cows varies during the year, as the CR decreased in spring/summer compared to the autumn/winter, albeit the S/C did not change seasonally. Since the mechanism by which thermal stress compromises fertility in dairy cattle is multifactorial, additional variables such as parity and daily milk production will be included in this study.

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AI / TAI / FTET

Testosterone, ano-genital distance, and epididymal sperm quality in bulls derived from artificial insemination and in vitro production

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Previous studies have documented variances in cattle born from artificial insemination (AI) and in vitro derived embryo transfer (IVP). However, it remains uncertain whether these variances are associated with testosterone levels and bull reproductive function. This study aims to investigate plasma testosterone levels in AI and IVP-derived bulls and to assess physical weight and length and morphological reproductive parameters, including anogenital distance, and epididymal sperm quality. Bulls born via AI (n=5) and IVP (n=9) previously produced (Lopes et al. Animals, 2020) were kept under the same housing, management and feeding conditions. They were evaluated at intervals 75, 150, 360, 600-900 and 1200-1500 days of age. Physical parameters (weight, length, height at withers, thoracic girth) and morphological reproductive parameters such as anogenital distance were measured. Average daily weight gain (ADWG) was calculated from birth. Blood plasma was collected and stored (-80ºC) for testosterone analysis, conducted using an automated clinical chemistry analyzer (Olympus AU400, Japan). Bulls were sacrificed at an age exceeding 1500 days and epididymal spermatozoa were collected for evaluation of motility (using CASA), viability (PNA-FITC and propidium iodide staining, plus flow cytometry), and morphology (contrast phase microscope evaluation). Data were analyzed using a mixed linear model with age and embryo origin (IVP *vs.* AI) as the main factors. Pearson correlations were computed between variables, with significance set at $P < 0.05$.

No significant differences were observed between AI and IVP-derived bulls regarding testosterone levels, physical parameters, or anogenital measurements ($P > 0.05$). Similarly, epididymal sperm parameters, including total motility (AI: 88.8 ± 4.1, IVP: 90.4 ± 5.3; %), progressive motility (AI: 53.2 ± 5.8, IVP: 40.0 ± 5.4; %), viability (viable and non-reacted sperm; AI: 78.9 ± 5.8, IVP: 82.1 ± 1.6; %), and morphology (percentage of abnormal sperm; AI: 93.5 ± 3.0, IVP: 89.0 ± 3.2; %), did not show significant discrepancies. Testosterone levels exhibited an increase with age, ranging from a mean of 13.6 ng/ml at 75 days to 740 ng/ml at 1200-1500 days. Both testosterone levels and age demonstrated direct and significant correlations with all physical parameters (body weight and length parameters) and anogenital distance. Age exhibited an inverse relationship with ADWG. In conclusion, within the constraints of this study's sample size, no disparities were detected in testosterone levels or epididymal sperm parameters among bulls derived from AI and IVP.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Factors influencing calving rates after transfer of frozen-thawed in-vivo bovine embryos considering quality of corpus luteum of recipient animals

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The main objective of this study was to evaluate the influence of a cavity in the corpus luteum (CL) of recipients on the success of embryo transfer. Other authors reported no differences in establishment of pregnancy with regard to cavity of the CL (Nogueira et al.,2012; Thomson et al.,2021). In one study (Jaśkowski et al.,2021), significantly better pregnancy results were achieved in recipients with a cavity in the CL than with a compact CL.Calving rates (CR) after the transfer of 330 in-vivo embryos, frozen in ethylene glycol from Holstein donors in North-West Germany between February and December 2022 were evaluated. Stage and quality of the embryos were assessed according to the IETS standard. The recipients were nulliparous animals (n=330) of the Holstein breed.The transfers took place 7 days after estrus on 64 different farms (1 to 46 transfers per farm).The recipient animals had to have a CL of at least 2 cm diameter on one of the ovaries. The CLs were divided into two categories: 1. Compact CL. 2. CL with a cavity. Size of the cavity was recorded but not accounted for in the analysis. Animals with a large follicle (>15 mm) on one of the ovaries were excluded. The diagnostics were carried out using EasyscanTM (IMV Imaging) ultrasound devices. The calving was evaluated using the SERVIT database (VIT,Verden).

The statistical analyses were carried out using SPSS. A multivariable mixed logistic regression model was created with calving (Y/N) as the outcome variable and embryo quality (1/2), embryo stage (4-7), quality of CL (1/2) and whether the animal was inseminated before embryo transfer (Y/N) as fixed effects. Interactions of embryo quality and stage with quality of CL were tested in the model but then removed due to non-significant effects. Herd was considered as a random effect (covariance type = variance components). A compact CL was found in 233 (70.6%) animals selected for transfer. A cavity of up to 50% was diagnosed in 88 animals (26.7%) and a cavity of over 50% was found in 9 animals (2.7%). The 330 transfers resulted in a total of 153 calvings (46.4%), in which 142 calves were born alive (92.8%) and 10 were born dead (6.5%). The mixed logistic regression model showed that neither embryo stage (P = 0.347), embryo quality (P = 0.573) nor CL quality (P = 0.755) had a significant influence on CR. Only prior insemination was significantly associated with CR (P = 0.011). The predicted means for CR were 27.7% (SE = 6.9) in animals that received an insemination before embryo transfer compared to 45.5% (SE = 6.5) in animals that did not receive prior inseminations. Predicted means for CR depending on CL quality was distributed as follows: Compact CL 35.2% (SE = 6.4), CL with cavity 37.0% (SE = 7.2). Transfers with stage 6 had the highest predicted CR with 46.6% (SE = 8.6). Transfers with stages 4 or 5 embryos resulted in predicted CR of 36.8 (SE = 5.7) and 36.5% (SE = 8.4) and stage 7 had a predicted CR of 25.9% (SE = 10.3). Quality 1 embryos had a predicted CR of 38.8% (SE = 4.9) and quality 2 embryos a predicted CR of 33.6% (SE = 9.4). Our analyses were able to confirm other studies in which a luteal cavity did not have a significant effect on calving rates.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Superovulatory response of Creole ewes treated with autologous platelet-rich plasma (PRP)

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The response to superovulatory treatments is usually low and variable, related to factors such as hormones used, dosage and administration, but also to animal and environmental factors. The application of PRP increases the follicular population ≥2 mm in ewes. However, there is no information on the response to superovulatory treatments. Therefore, attempts to improve responses are justified. The study was carried out on the experimental farm at Universidad Autonoma Chapingo, Mexico with 20 multiparous, healthy Creole ewes with good body condition. The ewes selected at random from a bigger group and without reproductive problems. PRP was obtained according to the procedures defined by Sanchez-Chavez (2023). Platelet concentration was adjusted to 12 500 × 10^6 platelets mL^{-1}. The experimental design was completely random, and the treatments were: T1 = (Control) left ovary received 50 µL of saline solution (n=10) and T2 = right ovary treated with 50 µL of PRP (n=10), in both cases administered through laparoscopy. PRP application on right ovary was because preliminar study did not show significant effect of ovary to PRP administration. The population of ≥2 mm follicles in both ovaries, with an ultrasound (Aloka, Prosound 2, Japan) and a rectal transducer (7.5 MHz) was determined at the beginning of the study and four weeks after PRP administration. The ewes followed a synchronization protocol by inserting an intravaginal releasing progesterone device (CIDR, Zoetis, Mexico) for 12 days, on day 10 they were superovulated by administering 1 250 units of eCG (Novormon 5000, Zoetis, Mexico) as a single intramuscular injection and on day 12 the device was removed. Heat detection was conducted every 12 hours from 24 to 48 hours after CIDR removal with the help of a vasectomized teaser ram. The ewes were inseminated through laparoscopy 18 hours after estrus onset with two doses of fresh semen with 100 × 10^6 spermatozoa per dose, placing every dose in the middle part of each uterine horn, using semen from a proven fertility Creole ram. The superovulatory response was determined seven days after estrus through laparotomy using surgical standard procedures. The variables recorded were: initial number of follicles (INF), number of follicles after PRP application (NFAPRP), number of corpora lutea after estrus (NCL), number of large anovulated follicles (NLAF), and total ovulatory response (TOR= NCL+ NLAF). Data are presented as mean ± standard error. The GLM procedure of SAS was used to analyze the results, considering a significant effect with a $P< 0.05$. The NFAPRP, NCL, and TOR were higher in ovaries receiving PRP compared to ovaries treated with saline solution (3.94 ± 0.31 vs. 2.94 ± 0.31; 4.20 ± 0.60 vs. 2.42 ± 0.60; 5.42 ± 0.69 vs. 3.36 ± 0.69, respectively). However, there were no significant differences between treatments for the INF (3.05 ± 0.33 vs. 3.68 ± 0.33) and NLAF (1.21 ± 0.30 vs. 0.94 ± 0.30), respectively. In conclusion, the intraovarian administration of autologous PRP in Creole ewes allows a significant increase in the number of antral follicles ≥2 mm, the number of corpora lutea following a superovulatory treatment, and the total ovulatory response, under the conditions of this study.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Semen quality of young bulls does impact success rate of In Vitro Fertilization

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Shortening the generation interval is one important tool to increase the genetic gain within a breeding program. This can for instance be achieved by using semen of very young bulls (i.e. 10 months old) for In Vitro Fertilization, since it requires only a very limited amount of semen. However, the quality of the semen of young bulls could still be immature and therefore, results with respect to embryo development in In Vitro embryo Production (IVP) can be very variable.

The aim of this study was to see if semen quality is affecting the IVP embryo development and if selection of the semen based on quality is useful to improve IVP results.

Semen quality data (motility and morphology) and embryo development data were available for 3108 different fresh ejaculates of 142HF bulls in the age of 10-12 months. Ejaculates were collected using an artificial vagina and according to the standard CRV protocol. The percentage motile cells was measured by the AndroScope CASA system (Minitube, Tiefenback, Germany) and the percentage morphological abnormal cells was determined by staining a sample using eosin/analin and microscopically counting 100 sperm cells (1000x magnification), differentiating in eight categories (normal cells, three primary and four secondary type of morphological abormalities). In the 3108 OPU sessions on average 10 oocytes were used (min-max; 2-43 oocytes). A total amount of 2,0 x 10^6/ml of semen was used to fertilize oocytes collected from dams in the age of 12-13 months. Embryo development (defined as the % of Q1+2 Day 7 embryos based on number of oocytes in IVC) was monitored using OPU derived oocytes after maturation, fertilization (with the different batches of fresh semen) and culture for 7 days, using the standard CRV protocol. Data were analysed using regression and Chi-square analysis.

The results from the regression analysis between the semen quality parameters and embryo development all showed a very low, non-significant correlation (P>0.05). But when both percentage morphological abnormalities and percentage motility were divided in two classes (below 50% and 50% or higher), a clear difference was observed in the embryo development. Using semen samples (n=273) that contained 50% or more morphological abnormal cells results in a significant (P< 0.001) lower embryo development rate compared to semen samples (n=2836) with less than 50% morphological abnormalities, i.e. 11% and 22% respectively. Samples with >50% abnormal cells varied in type of abnormalities, but mainly it involved non-compensatable traits like cells with a damaged acrosome, abnormal head shape, or containing proximal droplets. In addition, using semen samples with a motility of less than 50% (n=117) resulted in a significant lower embryo development compared to those (n=2992) with a motility of 50% or higher, i.e., 13% and 22% respectively. Combining both morphology and motility did not further change the results, since most samples with a high percentage of morphological abnormalities also had a low motility.

It is therefore concluded that semen morphology or motility of fresh semen samples is affecting the In Vitro embryo development and that these quality parameters can be used to eliminate semen batches that give a low embryo development.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Capacitation-IVM system with C-type natriuretic peptide affects mitochondrial distribution, and calcium levels and patterns in lamb oocytes

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In vitro matured oocytes of prepubertal females present a lack of synchronization between cytoplasmic and nuclear maturation (Kochhar et al., Reprod Dom Anim 37:19-25, 2002). Biphasic IVM systems that use C-type natriuretic peptide (CNP) to prevent germinal vesicle breakdown before inducing oocyte in vitro maturation has been called Capacitation-IVM (CAPA-IVM). This study aimed to assess the effect of CAPA-IVM system on lamb oocyte quality. Ovaries from 1-5 months old lambs were recovered at a local slaughterhouse. Cumulus-oocyte complexes (COCs) were collected by slicing and selected using HEPES-buffered TCM-199 medium with the meiotic inhibitor 3-Isobutyl-1-methylxanthine (500µM) and heparin. COCs were cultured in a pre-maturation medium (TCM-199, 4mg/mL BSA, 0.2mM sodium pyruvate, 1mM glutamine, 100 µM cysteamine, and 5µg/mL gentamicin) with 20nM CNP and 10nM 17ß-estradiol (E2), for 6h and in conventional IVM medium (TCM-199 with 0.2mM sodium pyruvate, 1mM glutamine, 100µM cysteamine, 5µg/mL LH, 5µg/mL FSH, 3.7µM E2, 10ng/mL EGF, 10% FBS, and 5µg/mL gentamicin) for further 24 h (CAPA-IVM group). The control group was cultured in a conventional IVM medium for 24 hours. After 24 h (control group) and 30 h (CAPA-IVM group) of culture, 30 COCs per group were denuded and 10 oocytes were stained with Mitotracker Orange CMTMRos (Molecular Probes, Eugene OR, USA) to analyze the mitochondrial distribution following the patterns described by Martin Maestro et al (Animals 10:2414. 2020); 10 oocytes were stained with a commercial Calcium stain kit (Abcam, Cambridge, UK) to check the distribution and the level of calcium by measuring the intensity of fluorescent signal; and 10 oocytes were used to measure the early apoptotic using the annexin V-FITC apoptosis staining kit (Abcam). Three replicates were made. Images were taken using a optical microscope camera and analysed with ImageJ program. Data were statistically analyzed by Fisher's exact test (cualitative data) and multiple T-test (cuantitative data). The data obtained from the Mitotracker stain showed 1.5 times more intensity ($p<0.0001$) and a higher percentage of normal distribution pattern in CAPA-IVM group than in control group (76.7% and 60.0%, respectively; $p=0.0149$). The calcium stain showed that calcium levels were 2.9 times higher ($p<0.0001$) in CAPA-IVM group than in control group and 73.33% of oocytes from CAPA-IVM group presented a peripheric distribution (under the plasmatic cell membrane) while only 30% of oocytes from control group showed this distribution ($p<0.0001$). Finally, CAPA-IVM system and conventional IVM protocol provided similar percentages of early apoptotic (10% and 13%, respectively; $p=0.687$) and dead (3% and 17%, respectively; $p=0.097$) oocytes.

In conclusion, the results from this study show that CAPA-IVM system increases the quality of the oocyte from prepubertal donors via increasing the number of mitochondria and its normal distribution, increasing the intracytoplasmic calcium and showing a peripherical distribution that can influence avoiding polyspermy by the activation of the mechanism involved in the cortical reaction.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Concentration of progesterone, relative mRNA abundance of Interferon-Stimulated Gene-15 and concentration of Pregnancy-Specific Protein B in maternal circulation following timed artificial insemination or embryo transfer in lactating dairy cows

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The objective of this study was to provide early pregnancy diagnosis and examine the relationship between progesterone (P4) concentration, relative mRNA abundance of interferon-stimulated gene-15 (ISG15) and concentration of Pregnancy-Specific Protein B (PSPB) in maternal circulation following timed artificial insemination (AI) or timed embryo transfer (ET). Following estrous synchronization, lactating Holstein-Friesian cows received either AI on d 0 (n = 119) or ET on d 7 (n = 418) with a fresh or frozen in vitro-produced (IVP) blastocyst derived from oocytes collected by transvaginal ovum pick-up from n = 14 Holstein Friesian, n = 8 Jersey and n = 21 Angus donors. Blood samples were collected on d 7 (n = 537), d 18 (n = 524) and d 25 (n = 378) to determine P4, ISG15 and PSPB, respectively, to provide early pregnancy diagnosis and measure associations with subsequent probability of pregnancy or pregnancy loss (PL). Transrectal ultrasonography to determine pregnancy status occurred on d 32, d 62 and d 125 and parturition date was recorded. All available variables where P < 0.1 were included in the final model for analysis (PROC GLIMMIX, SAS). Cows with greater P4 on d 7 had greater ISG15 on d 18 (P < 0.0001) and PSPB on d 25 (P = 0.002). mRNA abundance of ISG15 on d 18 was positively associated with serum PSPB on d 25 (P < 0.0001), and both varied by serum P4 quartile (P = 0.01 and P = 0.007, respectively). There was no overall effect of treatment (AI vs fresh ET vs frozen ET) on mRNA abundance of ISG15 in cows that were pregnant on d 18 (P = 0.158), but there was a tendency for cows that received fresh ET to have greater ISG15 than cows that received frozen ET (P = 0.097). Conversely, a strong treatment effect was detected for serum PSPB on d 25 in pregnant cows (P = 0.0002). Cows that received AI and fresh ET had greater PSPB than cows that received frozen ET (P = 0.006 and P < 0.0001 respectively). Cows with greater mRNA abundance of ISG15 (d 18) and serum PSPB (d 25) had a greater probability of pregnancy (d 32, d 62, d 125) and of reaching full term parturition (all P < 0.0001). Day 18 ISG15 expression was negatively associated with PL after d 18 (P = 0.0139). The PL (%) for each quartile of ISG15 was: 47.2%, 39.3%, 43.6% and 22.4% for quartiles 1 to 4, respectively. Serum PSPB on d 25 was associated with the incidence of PL after d 25 (P < 0.0001). Cows in the lowest quartile for PSPB had greater PL (58.1%) than cows in PSPB-Q3 (19.2%; P = 0.023) and PSPB-Q4 (9.6%, P = 0.002). Cows in PSPB-Q2 (35.5%) had greater PL than cows in PSPB-Q4 (P = 0.0003) and tended to have greater PL than PSPB-Q3 (P = 0.051). In conclusion, cows that had greater P4 on d 7 had greater mRNA abundance of ISG15 on d 18, indicating a stronger maternal response to conceptus-derived interferon-tau. These cows also had a greater PSPB on d 25, which was associated with greater likelihood of reaching full-term parturition. This study highlights the temporal pattern of pregnancy establishment and loss in cows that were bred using AI or IVP-ET, and important areas for additional research to improve IVP procedures.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Cumulus-oocyte communication in prepubertal goats according to GV chromatin configuration and nuclear stage during in vitro maturation

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Oocyte competence for embryo development relies on bidirectional communication with cumulus cells (CCs) via gap-junctions located at the tip of transzonal projections (TZP). In cattle, functional gap junction-mediated communication decreased in oocytes with a high chromatin condensation in the germinal vesicle (GV), and it was related to early signs of atresia (Lodde, Mol Reprod Dev, 74:740-749, 2007). Sirard (Anim Reprod, 16:449-454, 2019) suggested that bovine oocytes with an intermediate level of chromatin condensation could be related to high developmental competence. Our aim was to analyze cumulus-oocyte communication, assessed by TZP density, according to GV chromatin configuration at the beginning of IVM and nuclear stage during IVM. Cumulus-oocyte complexes (COCs) were collected by ovary slicing and matured in TCM-199 with FSH, LH, estradiol, EGF and cysteamine during 24h at 38.5ºC with 5% CO2. Oocytes with two or more layers of compact cumulus cells and homogeneous cytoplasm were selected. A sample of 10 COCs/replicate (3 replicates) were recovered at several times during IVM (0h, 6h, 12h and 24h) for TZP density and nuclear assessment. COCs were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.25% triton for 30 min and actin filaments were stained with 5 µg/mL phalloidin-FITC for 1h. Then, COCs were counterstained with 1 µg/mL Hoescht 33258 for 10 min and analyzed with confocal laser microscopy. One image was taken per oocyte in the equatorial plane and TZP density was quantified as phalloidin-FITC average fluorescence intensity in the whole zona pellucida area. Oocyte GV chromatin configuration and nuclear stage was classified according to Sui et al. (Mol Reprod Dev, 71:227-236, 2005) as: GV1 (diffuse filamentous chromatin), GVn (condensed net-like chromatin), GVc (condensed clumped chromatin), GV breakdown (GVBD), metaphase I (MI) and metaphase II (MII). Twenty-five oocytes in GV at 0h, 11 oocytes in GVDB at 6h, 24 oocytes in MI at 12h and 21 oocytes in MII at 24h were analyzed. Data were statistically analyzed by two-way ANOVA followed by Tukey's correction. Results showed that, at 0h, GVn-oocytes presented a higher TZP density ($p<0.05$) compared to GV1 and GVc (20.65, 13.07 and 12.84 arbitrary units; n= 8, 9 and 8; respectively), which did not differ significantly from each other. At 6h, the TZP density of GVBD-oocytes (15.05 arbitrary units) did not differ from any GV configuration. MI-oocytes at 12h and MII-oocytes at 24h had a similar TZP density (7.98 and 4.03 arbitrary units; respectively), which was lower ($p<0.05$) compared to GVBD. In conclusion, TZP density in prepubertal goats increases with GV chromatin condensation up to an intermediate stage (GVn), then it declines when the oocyte reaches a high GV chromatin condensation. Following nuclear maturation, the oocyte and CCs progressively lost communication.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

The positive association between maternal body condition score and antral follicle count does not affect the number of produced blastocysts in OPU mares

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Little is known regarding the association between a mare's body condition score (BCS) and the ovum pick-up (OPU)-intracytoplasmic sperm injection (ICSI) outcomes. This study aimed to study the effect of maternal BCS on oocyte developmental competence and to detect if the BCS can determine the mare's ability to produce embryos *in vitro*. Overall, 107 OPU-ICSI sessions were performed in Warmblood and Quarter mares (*n*= 59), with a BCS of 2-8 out of 9. These mares were admitted to the routine commercial OPU-ICSI services at the Faculty of Veterinary Medicine, Ghent University. The OPU-ICSI outcomes, including the antral follicle count, the number of recovered oocytes, the number of mature oocytes, the number of presumptive cleaved zygotes, and the number of blastocysts were recorded per session. Spearman's correlation coefficient was checked between BCS and antral follicle count. For the blastocyst-producing sessions, the OPU-ICSI outcomes of thin (BCS= 2-4; *n*= 27), fit (BCS= 5-6; *n*= 33), and obese (BCS= 7-8; *n*= 5) mares were compared using Welch-One way ANOVA followed by the Games-Howell or Kruskal-Wallis test. For all mares, the BCS was significantly associated with the antral follicle count (*r*= 0.234, *P*= 0.019). For the blastocyst-producing sessions, the antral follicle count between thin, fit, and obese mares was significantly different (16.73 ±1.39, 22.58 ±1.61, and 22.60 ±8.63, respectively, *P*= 0.022), which did not significantly change the number of produced blastocysts (1.73 ±0.27, 2.39 ±0.36, and 2.20 ±0.97, respectively, *P*= 0.547). However, there were no differences (*P*> 0.05) in the numbers of recovered oocytes, mature oocytes, and cleaved zygotes between thin (11.23 ±1.07, 7.15 ±0.75, and 4.81 ±0.45, respectively), fit (14.58 ±1.46, 8.55 ±0.89, and 5.84 ±0.70, respectively), and obese (14.80 ±6.61, 9.80 ±3.72, and 4.20 ±1.32, respectively) mares. Taken together, the maternal BCS cannot determine the mare's ability to produce blastocysts after OPU-ICSI. The blastocyst-producing thin mares (BCS= 2-4 out of 9) exhibited a lower antral follicle count, which did not affect the final number of produced blastocysts between thin, fit, and obese mares.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Interleukin-6 supplementation during in vitro maturation improves the spindle and chromosome organization of pig oocytes

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Interleukin-6 (IL-6) is a multifunctional cytokine which, present in follicular fluid, is involved in oocyte maturation. Since this effect on pig oocytes in vitro matured (IVM) is uncertain, this study evaluated the impact of adding IL-6 to the IVM medium on the maturation rate and the organization of chromosomes and spindle during meiotic maturation. For this purpose, oocytes collected from ovaries of prepuberal gilts of 6 months of age were matured for 44 hours in IVM medium consisting of TCM-199 with 0.57mM cysteine, 0.1% PVA and 10 ng/mL epidermal growth factor and supplemented with 0 (control; N=182), 10 (N=152), 50 (N=141), 100 (N=136) and 200 (N=164) ng/mL of IL-6. Following the IVM, oocytes were denuded, fixed, and stained with Hoechst 33342 and anti-α-tubulin antibody conjugated with FITC to assess maturation and the percentage of aligned chromosomes and well-formed spindle. Subsequently, the oocytes were examined under fluorescence microscopy (Hoechst 33342: blue fluorescence; α-tubulin-FITC: green fluorescence). The maturation rate was calculated as the percentage of oocytes with a metaphase plate and a polar body over the total number of oocytes. The proportion of well-aligned chromosomes was determined as the ratio of oocytes with an organized structure (forming a line or ring) to the total number of oocytes evaluated. Conversely, the proportion of well-formed spindles i.e, the ratio of oocytes displaying the spindle with a bipolar morphology (barrel-like shape) or a well-structured network of microtubules (star-like shape), was considered over the total number of oocytes. The experiment was replicated 4 times. Maturation rate data were analyzed by a mixed ANOVA model followed by the Bonferroni post hoc test, and the results presented as means ± SD. Data on aligned chromosomes and well-formed spindle were analyzed using the Chi-square test, and the results presented as percentages. While no differences were observed among maturation rates of oocytes treated with IL-6 compared to controls (range from 83.2±31.5% to 89.3 ± 8.6%), oocytes treated with 100 ng/mL of IL-6 showed a significant increase ($P< 0.05$) in the percentage of aligned chromosomes (65.6%) and well-conformed meiotic spindle (66.4%) compared to controls (53.2% and 52.4%, respectively). These findings indicate that the addition of IL-6 to the IVM medium of oocytes does not increase maturation rate. However, exogenous 100 ng/mL of IL-6 significantly improves the morphology of metaphase plates and spindles of IVM-prepubertal pig oocytes, a crucial aspect for producing viable oocytes and influencing their future development.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Effect of embryo breed and type of semen used for IVF on gestation length and calving characteristics following fresh transfer of IVP embryos

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The aim of this study was to compare gestation length (GL), calving difficulty (CD) and birth weight (BW) of calves derived from in vitro produced (IVP) embryos from dairy and beef breeds. Oocytes were collected once per week for up to four successive weeks from Holstein-Friesian dairy donors (HF, n = 51) and Angus beef donors (AA, n = 34) using transvaginal ovum pick-up. Following in vitro maturation, in vitro fertilization was undertaken using either conventional (CV) or X-sorted (SS) sperm. On day 7, embryo recipients (n = 471) were randomly assigned to receive a single Grade 1 blastocyst (HF or AA) that had been fertilized with CV or SS. The embryo recipients were lactating dairy cows in seven herds that had been synchronized with a progesterone-Ovsynch protocol. Embryos were transferred fresh to the uterine horn ipsilateral to the corpus luteum. At the end of the subsequent gestation, dams were induced at day 280 of gestation if parturition was not imminent (n = 31). Parturition data were captured for up to 156 calves on 6 herds (HF, n = 58, AA, n = 98). Individual calf data was available for GL (n = 156), CD (n = 130) and BW (n = 148). BW and CD were recorded immediately after calving, with CD being scored on a scale of 1 to 4: 1 = normal unassisted calving (n = 87), 2 = some assistance (n = 32), 3 = considerable assistance (n = 9) and 4 = veterinary assistance (n = 2). Treatment effects on BW, GL and CD were determined using generalised linear mixed models in SAS. The first analysis included calves sired by 7 HF bulls and 6 AA bulls, and a second analysis was restricted to bulls that had calves derived from both CV and SS semen (HF, n = 5, AA, n = 4). In the first analysis, embryo breed, semen type, and induction status were included as fixed effects. The second analysis also included the effects of sire and sire x semen type interaction. BW was greater for AA calves compared with HF calves (42.9 kg vs 37.6 kg, P < 0.001) but tended to be less in calves generated using CV compared with SS (39.2 kg vs 41.4 kg, P = 0.073). GL was not different for AA compared with HF calves (280.9 ± 0.5 days vs 280.6 ± 0.6 days, P = 0.57) or between calves generated using CV or SS sperm (280.9 days vs 280.6 ± 0.5 days, P = 0.65). The odds of a difficult calving was not affected by breed (HF vs AA, OR = 0.94, 95% CI = 0.40, 2.20, P = 0.88) or semen type (CV vs SS, OR = 1.32, 95% CI 0.57, 3.05, P = 0.50). Cows that were induced had increased odds of calving difficulty (Induced vs Spontaneous OR = 8.45, 95% CI 3.47, 20.57, P < 0.001). In the second analysis, the interaction between sire and semen type was not significant for GL, BW or CD (all P > 0.12). Sire had an effect on BW (P = 0.015) and CD (P = 0.002), but not GL (P = 0.22). In conclusion, calf BW was affected by breed but not semen type, GL was not affected by breed or semen type and CD was not affected by breed or semen type but was affected by induction status.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Vitrification by two different protocols does not affect pregnancy outcomes in in vitro-produced equine embryos.

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Despite the fast evolution of in vitro production in horses, there is still limited understanding of the impact of the effect of different vitrification protocols and the transfer of older embryos (i.e. > 10 days to become embryos) on pregnancy outcomes. Here, we aim to evaluate the effect of the vitrification method and the developmental kinetics on pregnancy rates and embryonic loss. To do so, equine embryos (n=139) were produced after ovum pick-up and intra-cytoplasmatic sperm injection in a commercial program. Day of blastocyst formation was evaluated and categorized as early (day 7 and 8; n=52), mid (day 9 and 10; n=65) and late (day 11 to 13; n=22), and transferred fresh (n=28) or vitrified on a Cryolock (Irvine Scientific) by one of the following protocols: (1) holding of embryos in base solution (BS; DMEM-F12 with 20% FCS), vitrification for 5 min in solution 1 (VS1; BS+ 1.5M ethylene glycol) followed by 40 s in solution 2 (BS + 7M ethylene glycol and 0.6M galactose); (2) vitrification at 23°C with a commercial VIT-Kit (Minitube, Germany) in VS1 (5 min), VS2 (5 min) and VS3 (40 s), containing increasing concentrations of glycerol and/or ethylene glycol, based on (Eldridge-Panuska et al. Theriogenology. 2005; 63:1308-1319). Subsequent warming was performed in three-steps for protocol 1: thawing medium (TM; DPBS 0.1% glucose, 36 mg/L pyruvate, 0.4% BSA) with 0.3M sucrose (1 min), 0.15M sucrose TM (5 min), and holding in TM (5 min) at 38.2˚C (Choi and Hinrichs. Theriogenology. 2017; 87:48-54); or directly at 38°C for 5 min in Emcare holding medium (Spervital, The Netherlands) in protocol 2. All embryos were washed four times in Emcare and transferred immediately to a day 4 recipient mare. Early pregnancy (7d post transfer), and embryonic loss (loss of pregnancy between first positive control until 42d of gestation) were registered. Generalized mixed-effects models were used to test the effect of the vitrification method and embryo developmental category on pregnancy outcomes. Similar early pregnancy was obtained with fresh (71.4±8.5%) or vitrified embryos (both methods; 68.9±4.24%; p=0.8), so was between fresh and the three-step warming (69.5±6.0%) or the direct warming (68.3±6.0%). Likewise, similar embryo loss rates were observed by comparing fresh (20.0±8.9%) with three-step (31.7±7.3%; p=0.6) or direct warming (12.2±5.1%; p=0.7). However, there was a tendency in favor of direct against the three-steep warming (p=0.09). As vitrification did not affect pregnancy outcomes, the effect of blastocyst kinetics was evaluated over all embryos (fresh and vitrified). No differences were found in pregnancy rates or embryo loss between early (76.9±5.8% and 12.5±5.2%, respectively) and mid embryos (72.3±5.5% and 17.0±5.4%; p=0.9).However, late embryos tended to reduce pregnancy rate (45.5±10.6%) against early (p =0.05) and mid (p=0.1), and exhibited higher embryo loss (30.0±14.4%, p<0.05) compare to both groups. In conclusion, vitrification is a safe method to preserve equine embryos with flexibility using protocols with direct warming, and the transfer of old embryos (>d10) should be analyzed carefully to avoid pregnancy losses.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Comparative analysis of in vitro production efficiency between adult sows and prepubertal gilt ovaries

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The in vitro production of porcine embryos has become a pivotal technique in reproductive biology and biotechnology laboratories, serving not only in animal breeding programs, but also in the generation of models for human disease research, due to the genetic and physiological similarities between pigs and humans. For these reasons, establishing a standardized protocol to enhance the IVP of porcine embryos is of paramount importance. This goal can be attained by standardizing the origin of gametes, the culture media and the techniques utilized for IVM, IVF, and embryo culture.

In this sense, we have avoided the source of variation between males and ejaculates by employing frozen-thawed boar semen straws. We have also developed a complete set of ready-to-use, lyophilized culture media for pig IVP embryos, which can be stored for long periods, minimizing batch-to-batch variation and mitigating the risk of formulation errors associated with homemade media preparations. Nevertheless, achieving an optimal source of oocytes remains a challenge, including limited access to reproductive material at local abattoirs and the reduced developmental potential of immature oocytes from prepubertal gilts. To identify the optimal oocyte source, we analysed the variation in the IVP yield between ovaries sourced from prepubertal gilts and adult sows collected simultaneously from the same abattoir.

Ovaries were collected from adult sows (S) and prepubertal gilts (G), the latter selected based on ovarian morphology (absence of corpora lutea or albicantia), at a local abattoir. Following collection, COCs were aspirated from follicles of 2-8mm, selected based on morphology, and cultured for 22h in NaturARTs-PIG-IVM1-LYO medium (EmbryoCloud, Spain), and 22h in NaturARTs-PIG-IVM2-LYO medium. Following IVM, frozen-thawed boar sperm, same boar and ejaculate, was selected by swim up in PIG-SUM-LYO medium, and oocytes and spermatozoa were coincubated for 22h in PIG-IVF-LYO medium, after which embryos were transferred to PIG-IVC1-LYO medium for 24h, when cleavage was evaluated. After this, 2-cell embryos were transferred to PIG-IVC2-LYO medium until day (d) 7 of culture. Embryo stage was observed on d6 and d7 after insemination. A total of five replicates were conducted, with 100 oocytes per group per replicate. Statistical analysis was performed using Student's t-test ($P < 0.05$).

The cleavage rate (%) on d2 was significantly higher in S (77.5±1.9) compared to G (67.4± 2.1). Similarly, blastocyst formation rate (from cleaved embryos, %) on d6 was higher in S (55.6±2.6) than in G (28.7±2.5), with a consistent trend observed on d7 (S: 50.6 ±2.6; G: 29.9±2.5). There were no significant differences in blastocyst expansion rates between groups (16.0± 3.8 and 14.0± 2.5 on d6; 34.7±4.8 and 33.5± 3.5 on d7; for G and S, respectively).

Our findings suggest that ovaries from adult sows yield significantly higher numbers of embryos with similar developmental kinetics compared to prepubertal gilts. Thus, for both biomedical applications and genetic improvement in swine breeding programs, adult sow ovaries may be preferable for in vitro embryo production.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Relationship between fetal morphometrics and birth weight of calves following the transfer of in vitro-produced embryos derived from conventional or sex-sorted sperm

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The ability to accurately predict calf birth weight during gestation would be of significant benefit to dairy farmers, improving both cow and calf welfare. Data on fetal development and calf birth weight (BW) following in vitro fertilization with sex-sorted semen remain scarce, however. The aim of this study was to investigate the effect of semen type and embryo genotype on fetal size at Day 63 of gestation and calf BW of female calves following timed embryo transfer with fresh in vitro produced (IVP) embryos. These embryos were produced using oocytes collected from either beef or dairy donors and fertilized in vitro using either conventional (CV) or X-sorted (SS) sperm. Single Grade 1 blastocysts were transferred fresh to recipient lactating dairy cows (n=510) in 7 herds. For all cows that had not returned to estrus, pregnancy status was diagnosed on Day 32-38 by transrectal ultrasonography. All cows diagnosed pregnant (n=277) were re-examined on Day 63; in pregnant cows (n=243, CV-Dairy=45, SS-Dairy=58, CV-Beef=67, SS-Beef=73), fetal sex (males, n=70, females, n=173) was determined based on the position of the genital tubercle. The sex ratio was 117:14 (F:M) when SS sperm was used, reflecting the accuracy of the sorting process. Only female fetuses/calves were considered in this study. A video of each fetus was recorded, and thoracic diameter (TD, n=106) and biparietal diameter (BPD, n=93) were subsequently measured using ImageJ's freehand line function. Calf BW (n=119) was recorded at parturition. ANOVA was conducted using PROC GLM in SAS to determine the effects of semen type (CV vs. SS), embryo genotype (beef vs. dairy), and two-way interactions on Day 63 TD and BPD, and calf BW. The relationship between BPD and TD and between each BPD or TD and calf BW was determined by Pearson correlation with PROC CORR. At Day 63, the mean (± standard deviation) TD and BPD for female fetuses was 18.8 ± 1.03 and 13.5 ± 1.03 mm, respectively, and it was not affected by semen type or embryo genotype. Beef heifers were heavier than dairy heifers (42.9 ± 7.78 vs 36.7 ± 7.05 kg; respectively, $P<0.0001$). There was a tendency for an interaction between embryo genotype and semen type ($P=0.06$), since beef heifers conceived with SS were heavier ($p<0.01$) than those conceived with CV, or dairy heifers conceived with both types of semen (43.8 ± 7.95, 39.0 ± 6.08, 37.0 ± 7.28 and 37.8 ± 7.73 kg, for SS-Beef, CV-Beef, SS-Dairy and CV-Dairy, respectively). BPD was not correlated neither with TD nor calf BW. However, TD was associated with calf BW ($R^2=0.25$, $P=0.02$). In conclusion, under the conditions of this study, TD is a better predictive measurement for the BW of female calves compared with BPD. Future studies will correlate Day 63 fetal measures and calf BW with maternal blood transcriptome analysis.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPEROVULATION / OPU-IVP / ET

Bovine donors infected with Epizootic Hemorrhagic Disease: Impact on in vitro embryo production and risk of transmission through in vitro embryos production

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Epizootic Hemorrhagic Disease Virus (EHDV) is an Orbivirus transmitted by insects (*Culicoïdes* spp). Although infected animals may remain asymptomatic, the most characteristic symptoms include ulcers and inflammation of the muzzle, ptyalism (stomatitis), lameness, weight loss, anorexia, and fever. This viral disease emerged in Europe in late 2022 (Spain, Italy) and more recently in France (autumn 2023) (ANSES, 2023). The aim of this study was to characterize the *in vitro* embryo production efficiency of asymptomatic EHDV-infected donors and to assess the risk of virus transmission during the *in vitro* embryo production procedures.

Four asymptomatic EHD infected cow donors from two herds that had a clinical episode of EHD 3 to 4 months before OPU session and six negative EHD cow donors were included in this study. The EHD infected donors were positive for EHD virus in blood samples, 10 days before the OPU session, by RT-PCR test (IDGene™ EHDV Duplex) with a Ct (Number of PCR cycles to detect DNA virus) between 33-35. Donors were stimulated with decreasing pFSH doses (Stimufol; Reprobiol®, Belgium) twice daily for 3 days (dose: 300 µg). Cumulus oocyte complexes (COCs) were collected by OPU 12 to 24 h after the last FSH injection and *in vitro* matured using a standard IVM protocol. COCs were fertilized with frozen-thawed X-sorted sperm in modified Tyrode's bicarbonate buffered solution medium using different non-pre-tested bulls. Presumptive zygotes were cultured in SOF medium up to Day 7 at 38.5 °C in 5% CO2 and 5% O2 with maximum humidity. Grade 1 expanded blastocysts (IETS classification) were recorded on days 6.5 and 7. Embryo production was analyzed with ANOVA ($p<0.05$ was significant).

A PCR test (ID Gene™ EHDV Duplex) was used to detect a conservée séquence of all EHD virus serotypes in OPU collected medium (CM), maturation medium (MAT), Fertilization medium (FERT), Denudation medium (DEN), culture medium (CULT), the last three embryo washing media (LAV) and unfertilized and degenerated embryos (EMB) of EHDV positive donors.

For EHDV-positive donors, viral RNA was not detected by RT-PCR in association with any of the media collected during IVP (CM, MAT, FERT, DEN, CULT, LAV) or in unfertilized and degenerated embryos (EMB).

The total number of selected COCs for IVM did not significantly differ between EHD and Control groups (8.7±2.5 vs. 9.5±3.5). At D7, Grade 1 expanded blastocyst rates were not different between EHD and Control groups: 38.5±17.4 vs 50.9±17.7.

In conclusion, asymptomatic EHDV donors still viropositive in blood by RT-PCR with a Ct between 33-35 do not exhibit reduced efficiency in embryo production. It seems that the EHD virus is not likely to be transmitted by *in vitro* embryo production, 3 to 4 months post-infection. Further studies are needed to assess the effect of infection of donors by the EHDV on embryo production and the risk of transmission through *in vitro* embryo production procedures at earlier stages of the infection.Thematic Section: 40th Annual Meeting of the Association of Embryo Technology in Europe (AETE).

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS / OOGENESIS

Follicular response to the intraovarian administration of autologous platelet-rich plasma (PRP) in Katahdin ewes

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In the ewe, the growth of antral ovarian follicles as waves depends on the action of FSH and the number of such follicles affects the response to superovulatory treatments. The platelets contain a high concentration of growth factors, which have positive effects on the stimulation of endometrial and follicular growth. The objective of the study was to evaluate the number of antral follicles in the ovaries after the intraovarian application of PRP. The study was conducted at the Experimental farm of Universidad Autonoma Chapingo, Mexico. Eleven multiparous (2.1 ± 0.8) Katahdin ewes, healthy, and in good body condition (3.2 ± 0.3) were used during the breeding season of 2023. The study was conducted according to the guidelines of the Ethical Committee (No. CECBS23-13) of Universidad Autonoma Metropolitana, Mexico. Blood samples were collected from the jugular vein in 2.7 mL vacutainer tubes containing sodium citrate and were maintained at 4 °C during the procedure. The samples were centrifuged at 1 000 *g* for 30 min and in order to obtain the platelets, the serum obtained was centrifuged for a second time at 1 500 *g* for 10 min. The upper two-thirds of the tubes were discarded, keeping the lower third part with a higher platelet concentration (12 500 × 10^6 platelets mL^{-1}). Using a laparoscope (Rigid laparoscope, Storz, Germany), 50 µL of autologous PRP were administered inside the ovarian stroma of the right ovary of each ewe, while the left ovarian stroma received 50 µL of saline solution, without pinching follicles ≥4 mm or corpus luteum. The ewes were fasted from food and water for 18 h and anesthetized by intravenous administration of 2.3 mg of 10% xylazine (Procin, Pisa, Mexico) and 7.7 mg of ketamine (Anesket, Pisa, Mexico) per 10 kg^{-1} of body weight. The experimental design and the treatments were: T1 (Control) = ovary with an application of saline solution and T2 = ovary with an application of PRP. Next, the number of antral follicles ≥2 mm in size in both ovaries was determined weekly, for six weeks, using an ultrasound (US) device (Aloka, Prosound 2, Japan) equipped with a rectal transducer (7.5 MHz). Before each US session, the ewes were fasted. The number of antral follicles was analyzed with GLM of SAS. The effect of treatment was considered significant with a $P< 0.01$. The number of antral follicles in the ovaries was similar ($P> 0.05$) between PRP and control in weeks 0, 1, and 2 (6.36 ± 0.43 vs. 6.27 ± 0.30; 5.81 ± 0.53 vs. 5.81 ± 0.42, and 4.72 ± 0.23 vs. 4.90 ± 0.28, respectively). However, there were significant differences ($P< 0.01$) for weeks 3 (8.45 ± 0.41 vs. 6.09 ± 0.34), 4 (7.90 ± 0.62 vs. 5.63 ± 0.38), 5 (9.00 ± 0.52 vs. 5.72 ± 0.42), and 6 (8.45 ± 0.37 vs. 6.09 ± 0.46). The results suggest that the use of autologous PRP might be an alternative to complement superovulatory treatments in ewes, increasing the number of antral ovarian follicles. In conclusion, under the conditions of this study, the intraovarian application of autologous PRP increased the number of antral follicles in the ovaries of Katahdin ewes from week 3 to 6 post-treatment.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS / OOGENESIS

The effect of a maternal obesogenic diet on offspring oocyte mitochondrial ultrastructure at birth in an outbred mouse model

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Obesity induces oocyte mitochondrial dysfunction with ultrastructural damage (e.g. broken membranes, rose-petal structures), predisposing offspring to metabolic disorders (*Marei, Scientific Reports, 10, 2020; Keleher, Plos One, 13, 2018*). Mitochondrial ultrastructural abnormalities (e.g. elongated, increased electron density, mitochondrial degeneration) were also reported in ovulated oocytes of offspring born to diet-induced obese (OB) dams (*Xhonneux, Frontiers in Physiology, 14, 2023*). Recently, we detected similar alterations in oocyte mitochondrial ultrastructure in primordial follicles of adult offspring born to OB mothers (*Xhonneux, Plos One, 2024, under review*). However, it remains unknown if these alterations are inborn, i.e. present in offspring oocytes at birth. Yet, this information is crucial for optimal fertility management in daughters born to obese mothers. Therefore, we hypothesized that pups born to OB mothers already have altered oocyte mitochondrial ultrastructure in the primordial follicle pool at birth. An outbred Swiss mouse model was used to increase the pathophysiological relevance to humans.

Swiss dams (age 3 weeks) were fed control (C, n = 5) or OB (n = 5, high-fat, high-sugar) diets for 7 weeks, then mated with the same Swiss males fed a chow diet in a cross-over design. Dams stayed on the same diet during pregnancy. Pregnancies were monitored and litters were culled within 1h post-partum. One ovary of one female pup per dam was collected for ultrastructural analysis of oocyte mitochondria using transmission electron microscopy. At least 6 primordial follicles per ovary in one random midway ovarian section were examined. Ultrastructure of all oocyte mitochondria in the examined section was categorized, based on shape, density, abnormal membrane structures, vacuoles and clustering, and analyzed with generalized linear mixed models (total mitochondrial count: C = 1587, OB = 1820).

Newborn primordial oocyte mitochondria categorized as homogenous, vacuolated, clustered, or with abnormal membrane structures did not differ between treatment groups. However, maternal OB diet slightly reduced the proportion of oocyte spherical mitochondria (C: 88.28% ± 1.00, OB: 85.64% ± 1.11, $P = 0.020$), and tended to increase the proportion of elongated mitochondria (C: 10.59% ± 0.96, OB: 12.49% ± 1.07, $P = 0.061$). Maternal OB diet also reduced the proportion of newborn oocyte mitochondria with electron dense structures (C: 34.45% ± 2.18, OB: 22.66% ± 1.69, $P = 0.035$).

In outbred mice, pups from OB mothers do show subtle but significant differences in oocyte mitochondrial features in the primordial follicles at birth. However, the abnormalities reported in previous studies in adult mature offspring oocytes were not detected at the primordial stage in newborns. Mitochondrial elongation was limited, and the change in electron density was opposite to that detected at adulthood. Functional consequences of these changes are yet to be determined.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS / OOGENESIS

Acute and chronic effects of a high-fat high-sugar diet on the granulosa cell transcriptome. Insights from an outbred mouse model

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Continuous consumption of western-type diets (high in fat and sugar, HFHS diets) results in systemic metabolic changes including low-grade inflammation, lipotoxicity and oxidative stress. This impairs different tissue functions, and eventually leads to obesity and increased risk of metabolic disorders. Consumption of HFHS diet and the development of obesity have also been linked with reduced fertility, partially by impacting ovarian follicle development and oocyte quality. Previous studies have shown that diet-induced alterations in metabolism and tissue functions are dependent on the exposure time and occur in a multiphasic pattern [1]. Similar patterns were reported in the alterations in oviductal cell functions [2, 3], but the dynamics of diet-induced alterations in the ovarian follicles have not been previously described. The aim of this study was to determine the onset and progression of changes in the granulosa cell transcriptomic profile after starting HFHS diet feeding.

For that, 5 week (wk) old female outbred Swiss mice were fed a control (CTRL, 10% fat) or HFHS (60% fat, 20% fructose) diet for up to 16wk. Mice (n=34 per treatment per timepoint) were sacrificed at 3 days, 1wk, 4wk, 8wk, 12wk and 16wk. This was done during the follicular phase of the estrous cycle by applying the Whitten effect for 24h. Granulosa cells were collected by puncturing the antral follicles in L15 medium, then the cells were washed with PBS by centrifugation, snap-frozen and used for RNA sequencing. Differential gene expression analysis was performed as a series of pairwise comparisons at all time points using edgeR. Gene Set Enrichment Analysis was then done with GAGE and significant GO terms ($q<0.1$) were summarised using the “Reduce and Visualize GO” tool (REVIGO).

A few significant GO term annotations were detected already at day 3 (21 GO terms), the majority of which were also detected at wk1 (but not afterwards), together with the dysregulation of another 228 GO terms (245 in total at wk1). These acute changes were mainly related to endoplasmic reticulum and mitochondrial functions, translation, cell differentiation and cell signaling. No significant GO terms could be detected at wk4, which may indicate transient metabolic adaptations. Subsequently, the highest number of significant GO term annotations was detected at wk8 (495, including 103 of those detected at wk1). The majority of these annotations were also detected at wk12 (255 GO terms) and wk16 (126 GO terms). These chronic changes are related to cell metabolism, steroid biosynthesis, immune responses, autophagy, biogenesis, and mRNA processing.

The results show that the ovarian follicle microenvironment is sensitive to very short-term changes in the diet composition, and that the alterations in ovarian cell biological functions follow a time-dependent multiphasic cascade. Defining and understanding these progressive changes should increase the awareness needed to protect reproductive health and improve efficiency of preconception care interventions.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS / OOGENESIS

Reconstruction of immature bovine cumulus oocyte complexes for IVM

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The majority of retrieval human cumulus oocyte complexes (COCs) are subjected to the ICSI for fertilization in the infertility treatment program by assisted reproductive technologies (ART). However, approximately 15% of human oocytes are immature after oocyte denudation when induction of superovulation was applied. Human germinal vesicle oocytes are usually discarded from the ART program due to their low maturation rate and competence. It is known that cumulus cells (CCs) play an important role in maintaining the meiotic arrest of oocytes and achieving their competence by communication through the gap junctions (connexins 37 and 43). The restoration of lost contacts between CCs and denuded oocytes *in vitro* could improve oocyte IVM outcomes and increase the available gamete number for fertilization.

The study aimed to evaluate the effect of different culture systems on the restoration of gap junctions between CCs and oocytes and IVM outcomes.

The study utilized bovine CCs and oocytes as a model. Ovaries were obtained from the local slaughterhouse and 4-8 mm follicles were aspirated. Retrieval immature COCs (n=747) with more than 2-3 layers of CCs were randomly divided into groups: 0, immature COCs; 1, COCs matured in a drop under mineral oil; 2, denuded oocytes (DOs) matured in a drop under mineral oil; 3, DOs with CCs matured in a drop under mineral oil; 4, DOs with CCs matured in round-bottomed plates with cell-repelling surface; and 5, DOs with CCs matured in a hanging drop. All oocytes were matured in IVM medium (Stroebech, Denmark) during 22 hours, then maturation rate and RNA expression level of the *Gja1* (connexin 43) and *Gja4* (connexin 37) genes were assessed in the study groups. Three biological replicates were conducted for assessment of the genes' expression in each group.

Our study has shown that CCs was expanded in group 1 while in groups 3 and 5 CCs made partial aggregates with oocytes. In group 4, all CCs formed a whole rounded aggregate with oocytes in the center. The *Gja4* RNA expression level was higher in oocytes in all groups after IVM with the highest value in group 2 ($p<0.05$). The *Gja4* RNA expression level in CCs was increased in groups 3-5 after IVM, while in group 1 it did not have any changes ($p<0.05$). The *Gja1* expression in oocytes was upregulated after IVM only in group 2 while in CCs it was downregulated in all the groups where CCs were presented regardless culture system ($p<0.05$). The highest maturation rate was recorded in group 1 (76.2%), while in groups 2-5 it was 29.4; 62.3; 35.1 and 56.1%, respectively.

These results confirm that CCs are very important for successful oocyte maturation, and premature denudation may provoke changes in expression profiles of connexin 37 and 43 in oocytes. Perhaps such changes were a kind of compensatory effect in order to restore lost connections between oocytes and CCs. Adding CCs to DOs in a drop or hanging drop co-culture system improves the maturation rate. The use of plates with a round bottom and a cell-repelling surface leads to restoring the round shape of reconstructed COCs and may keep oocytes under meiotic arrest longer.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS / OOGENESIS

Single-cell DNA methylation sequencing reveals epigenetic alterations induced by bovine oocyte in vitro maturation

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Oocyte *in vitro* maturation (IVM) is a key step for the *in vitro* production of bovine embryos. However, there is a concern that this process may induce suboptimal developmental competence of bovine oocytes including epigenetic alterations. Currently, epigenetic profile of bovine oocyte IVM is relatively limited and inconsistent, probably arising from differences in the detection approach and experimental design used between studies. The aim of this work was to determine whether DNA methylome alterations are present in bovine oocytes that were *in vitro* matured and to identify conserved biomarkers across species. To achieve this, the results of this study were compared to those obtained in a similar study with *in vitro* matured porcine oocytes (unpublished data). The study was performed on 18 *in vitro* matured oocytes recovered from 2-8 mm follicles of abattoir-derived bovine ovaries and 28 *in vivo* matured oocytes collected by ovum-pick-up. Transvaginal aspiration was performed at 96–98 h after GnRH administration. The analysis of DNA methylation was performed by single-cell whole-genome bisulphite sequencing. Then, differentially methylated regions (DMRs, FDR < 0.05, FC > 0.1) were determined using the R package limma. Results showed that global DNA methylation profiles differed between *in vitro* and *in vivo* groups. Individual oocytes were clustered using Uniform Manifold Approximation and Projection analysis, which showed a clear separation within the *in vivo* group according to breed and age. The analysis of DMR identified a lower number of hypermethylated and hypomethylated regions in the IVM group, which were more frequent in variably methylated regions (VMRs), promoters, transcripts and imprinted genes. No differences were found in methylation of CpG islands of genes previously related to large offspring syndrome between groups. Regarding the effect on genomic imprinting, methylation was lower for IVM oocytes in the imprinted gene *CDKN1C* and higher in the *BEGAIN* gene when compared to the *in vivo* group. In addition, the analysis of genes that have been previously predicted for their possible function in the imprinting process showed a number of differences between the *in vivo* and *in vitro* group. For example, we identified lower methylation in *in vitro* matured oocytes in CpG islands of 5 "candidate" genes *(SEMA7A, ZNF575, ATP4B, PDGFA, COMP)* while only one was hypermethylated in the coding region of the *PLCL2* gene. Finally, we identified conserved differences in methylation related to IVM between bovine and porcine oocytes for 14, 8 and 3 genomic features in the transcripts, VMRs and promoters, respectively. The findings indicate that some of the epigenetic alterations are associated with suboptimal developmental competence of IVM oocytes. In conclusion, these results could help to improve this technique when employing *in vitro* production procedures in cattle.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS / OOGENESIS

Evaluation of the effect of three drugs on follicular development in water buffaloes (Bubalus bubalis) from an in vitro embryo production program

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One limitation of the buffalo *in vitro* production of embryos (IVP) is the number of oocytes and their developmental potential. Contradictory results have been reported regarding the effect of different sources of drugs, natural or recombinant, on the induction of follicular development. This work aimed to evaluate the effect of the administration of three different drugs on follicular development in an IVP buffalo program. Twenty healthy adult parous females located in Itatí (Corrientes, Argentina) were used. Ovarian mapping was performed using an ultrasound scanner (Mindray, DP30-Vet). Groups of five animals were divided in 4 treatments (TRT).On day 0, all animals received an intravaginal device (IVD) with progesterone + 2 mg of estradiol benzoate intramuscularly (IM). The IVD was removed on the day of Ovum Pick-Up (OPU). In TRT-1 (Control), OPU was performed on day 7. In TRT-2, 1050 IU of recombinant equine chorionic gonadotropin (eCG, FoliRec®) was administered IM on day 4 and OPU was performed on day 7. In TRT-3, 2500 IU of serum eCG (Ecegon®) was administered IM on day 4 and OPU was performed on day 7. In TRT4, recombinant follicle-stimulating hormone (FSH) (Cebitropin B®) was administered IM starting on day 4, 105 µg distributed in decreasing doses every 24 hours for two days (60 µg and 45 µg). Forty-four hours after the last application, the OPU was performed. Before OPU the numer of follicles was counted and classified according to their size: minor (≤3 mmØ), medium (4-8 mm Ø) or large (>8 mmØ). Oocytes were evaluated within the follicular fluid, graded I, II, III, IV and expanded according to the International Embryo Transfer Society (IETS, IL), and pooled for each TRTs. Oocytes were matured, fertilized and obtained embryos were cultured for 6 days. Data was compared using the Mann–Whitney or comparisons of proportions tests; a p-value < 0.05 was considered as statistically significant. The average number of follicles per animal before and after treatment was 13±3 and 9.6±3, respectively, with no significant differences between TRTs. There were significant differences in the number of follicles from 4 to 8 mm between TRT 1 (p=0.02), TRT2 (p=0.03), TRT3 (p=0.05) and TRT4 (p=0.03), showing an increase of 2.4 times compared to the number of follicles at the day of IVD insertion. No significant difference was found between the drugs in the number of oocytes and embryos between TRTs. A range of 20 to 24 oocytes were obtained, with 15.7%, 30%, 50.5%, 3,8% corresponding to GI-GII, GIII, IV and expanded oocytes respectively. There was an increase in the number of medium sized follicles in all TRTs as reported in the literature. But it is paradoxical from this study that the control group had an increase in the number of follicles, and a decrease in the total follicular population. It was not possible to demonstrate an effect of the drugs or protocols on the quality of oocytes and the number of embryos which in this species remain low. The authors believe that the groups are too small to compare the 4 different treatments. Future studies need bigger groups or fewer treatments in the same study to obtain more robust results.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

FOLLICULOGENESIS / OOGENESIS

M6a RNA methylation during in vitro maturation of sheep cumulus-oocyte complexes with different development competence

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M6a is an abundant epigenetic modification of mammalian mRNAs and is largely undescribed in mammalian oocytes and embryos. It regulates mRNA metabolism and affects maternal transcript decay prior to embryo genome activation, thereby potentially affecting developmental competence. RNA methylation dynamics are controlled by three groups of modulators that add (writers), remove (erasers) or bind to (readers) the methyl group to the N6 position of adenosine (Qin et al. Int J Mol Med. 2020).

We used a well-characterized differential model of developmental competence consisting of cumulus-oocyte complexes (COCs) of adult (2-4 years old) and prepubertal (30-40 days old) Sarda sheep, that show respectively high and low development competence. The differential model was previously studied in terms of in vitro developmental capabilities and morphological, cellular, biochemical and molecular aspects.

Aim of this work was to evaluate m6a methylation in the differential model of developmental competence in terms of gene expression of m6a modulators and m6a abudance.

COCs were recovered from ovaries of adult and prepubertal sheep. Oocytes and granulosa cells (GCs) were separately stored in CellProtect Reagent (Qiagen, Hilden, Germany) at germinal vesicle stage (GV) or after 24h in vitro maturation (IVM). Total RNA was isolated from pools of 10 oocytes and GCs from pools of 25 COCs, with RNeasy MicroKit (Qiagen, Hilden, Germany), reverse transcribed and used for gene-specific relative quantification by Real Time-PCR. RNA isolated from GCs was subjected to m6A quantification with EpiQuik™ m6A RNA Methylation Quantification Kit (EpigenTeck, Farmingdale, NY, USA). Data were analyzed with the General Linear Model ANOVA, considering the two factors “competence” and “maturation stage” and their interaction. Differences were considered significant when $p<0.05$.

Relative transcript abundance was similar in oocytes and GCs of different developmental competence at the GV stage. Conversely, after IVM, low competence COCs showed altered gene expression compared to high competence COCs, and specifically downregulation of *METTL3*, *METTL14*, *VIRMA*, *YTHDF3*, *YTHDC1* and *ALKBH5* ($p<0.05$) in oocytes and, upregulation of *METTL14,VIRMA* ($p<0.05$) and*YTHDF3* ($p=0.05$) in GCs. In accordance with a higher expression of the methyltransferases, colorimetric assay showed higher m6A levels in total RNA of GCs derived from low competence COCs after IVM ($p<0.05$). Data show that m6a RNA methylation is altered in low competence sheep COCs after IVM and suggest an involvement of the epitranscriptome in oocyte developmental potential.

This study contributes to the growing understanding of the oocyte epitranscriptome which is crucial for unravelling the mechanisms involved in the oocyte-embryo transition, shedding light on the impact of epigenetic RNA modification in mammalian reproduction and unexplored aspect of female infertility.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Phosphatidylserine on sperm head interacts with Annexin A5 on oviduct luminal cilia to form a sperm reservoir in pigs

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After insemination in mammals, a sperm subpopulation reaches the oviducts, where they bind to luminal epithelial cells in the isthmus to form a "sperm reservoir". Sperm binding to isthmic epithelial cells (IECs) is assumed to select sperm of high fertilizing ability and lengthen their lifespan until ovulation time. Expression of annexins (ANX) was reported on the surface of the porcine oviduct epithelium (Teijeiro *et al*. Mol Reprod Dev 76:334, 2009). However, the mechanisms of sperm reservoir formation are still largely unknown. The objective of this study was to explore the role of ANXA5, a protein that binds with high affinity to phosphatidylserine (PS; Jing J. Int J Mol Sci 25(5)2865, 2024), on formation of sperm reservoir in pigs.

Fresh ejaculated semen from different pools of 3-5 Pietrain boars (6-24 months) of proven fertility were used for all experiments. Sperm motility was analyzed by computer-assisted sperm analysis (IVOS II). Sperm acrosome status (PNA) and membrane integrity (propidium iodide) were analyzed by flow cytometry. Isthmic mucosa fragments (IMF) were collected from pre-pubertal gilts at slaughterhouse and epithelial spheroids (ES) produced *in vitro* as previously described (Schmaltz *et al*. Theriogenology, 219:116, 2024). A Tyrode-based non-capacitating medium (NCM) or a capacitating medium containing caffeine and albumin were used for ES co-incubation with sperm (1.10^6/mL, 30 min). Bound sperm density on ES (in number of sperm/mm^2) was determined using confocal microscopy after Hoechst staining. Recombinant porcine (rp) ANXA5 (RP1796S-100; Kingfisher Biotech) and soluble PS (P7769; Sigma) were added to co-incubation media. Mouse anti-ANXA5 and anti-PS antibodies (Sigma) were used for immunodetection. Three to six biological replicates with different pools of ES and semen were performed for each experiment. One-way ANOVA followed by Tukey post-hoc tests were used to analyze the data.

ANXA5 was immunodetected exclusively on cilia at the surface of IMF and derived ES. In addition, rpANXA5 from 0.01 to 10 µg/mL and PS from 0.01 to 1 µg/mL inhibited boar sperm binding to ES in a dose-dependent manner without decreasing sperm motility compared to controls. Pre-incubation of sperm but not ES with 10 µg/mL rpANXA5 was sufficient to inhibit sperm binding to ES. The rpANXA5 and PS were immunodetected on boar sperm heads in the acrosomal region. Compared with NCM, the capacitating medium increased within 30 min the proportions of live sperm positive for PS detection, bound to fluorescent rpANXA5, and the bound sperm density on ES. On the opposite, acrosome reaction decreased the proportion of PS+ sperm, their ability to bind to rpANXA5 and prevent their binding to ES.

Our data indicate that PS on boar sperm acrosomal region and ANXA5 on isthmic cilia play a role in the formation of the sperm reservoir. Sperm membrane remodeling at the time of capacitation enhanced sperm head PS exposure and may facilitate interactions with ciliary ANXA5 in short time. This is the first report of a role of PS-ANXA5 interaction and sperm capacitation in the formation of the oviduct sperm reservoir.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Enhancement of boar epididymal sperm motility through seminal plasma extracellular vesicles

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Extracellular vesicles (EVs) from sexual accessory glands have been implicated in various aspects of sperm function, including motility, acrosomal integrity and calcium mobilization. While epididymal vesicles interact directly with sperm in the male reproductive tract, EVs from sexual accessory glands are thought to influence sperm function in the female reproductive tract (reviewed by Foot and Kumar, 2021). However, studies on the role of accessory sexual gland EVs in sperm function and preservation have yielded conflicting results (Pons-Rejraji et al., 2011; Park et al., 2011). This study aims to investigate the effect of EVs from seminal plasma on the viability, acrosomal integrity and mitochondrial activity of epididymal spermatozoa during 72 h storage (time to preserve the insemination dose in porcine).

Seminal plasma from boars with proven fertility was used for isolation of EVs by ultracentrifugation from five different ejaculates. The EVs were characterised by means of DLS, TEM and EV-specific protein markers (CD63, CD81, HSP70) as previously described Toledo-Guardiola et al. 2024. To assess sample purity, high-resolution flow cytometry was performed to quantify the amount of albumin, a contaminant present in very small EVs. The same pool of EVs was used for all replicates (n=7). Epididymal spermatozoa from randomly selected boars from the abattoir were co-incubated with EVs or not (control group) and stored at 15ºC for up to 72 hours. Total and progressive motility parameters were assessed by Computer-Assisted Sperm Analysis (CASA), while flow cytometry was used to evaluate sperm viability (propidium iodide), acrosomal integrity (peanut lectin) and mitochondrial metabolism (rhodamine 123) at 0, 24, 48 and 72 hours.

The results showed that epididymal spermatozoa supplemented with EVs exhibited an increase in total motility (24 and 48 hours) and an increase in progressive motility (48 hours) compared to the control group. However, there was a deterioration in acrosomal integrity with vesicle supplementation at all time points. Viability and mitochondrial activity remained unaffected by the presence of vesicles but declined over the preservation period.

This study demonstrates the effect of EV supplementation of epididymal sperm on motility and acrosome integrity, which may be related to calcium mobility by ATPase contained in EVs. However, no work with epididymal spermatozoa has been found in the literature to support the results of this study.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Graphene oxide increases the sperm fertilizing ability by modifying the sperm proteome

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Over the last 30 years, our society has witnessed to an impressive increase in the adoption of in vitro fertilization (IVF) techniques due to the continuous rising incidence of infertility rates. To surpass the limits related to sperm selection, the use of non-physiological materials has emerged as one of the most promising strategies for advanced sperm preparation. Indeed, recent studies have reported that the addition of graphene oxide (GO) at specific concentrations during capacitation is able to increase the fertilization ability of spermatozoa in swine, bovine and mice models. On this basis, this study aims to elucidate the complex events occurring during sperm capacitation in the presence of GO, using a proteomic approach in a swine model. To this end, spermatozoa were analyzed at different capacitation times (0 and 2 h, T0 and T2 respectively) in the presence and absence of GO at 0.5 µg/mL (GO and CTRL groups, respectively). Proteomic analyses were performed on spermatozoa pooled from three different animals using Filter Aided Sample Preparation (FASP) protocol. Tryptic peptides were analysed in triplicate by LC-MS/MS using the UltiMateTM 3000 UPLC chromatographic system coupled to the Orbitrap FusionTM TribridTM (Thermo Fisher Scientific) mass spectrometer. Data were processed using MaxQuant and Perseus, matching spectra against the UniProt database (taxonomy Sus scrofa) to obtain LFQ Intensity values used for functional analysis. Finally, protein ratios (GO/CTRL T0 and GO/CTRL T2) were used for functional analysis by Ingenuity Pathway Analysis (IPA software, Qiagen, Hilden, Germany). In particular, 755 and 626 proteins were quantified in the GO and CTRL groups at T2, respectively, obtaining different protein sets with a total of 274 proteins with different values in terms of abundance ($p<0.05$). For instance, different values were obtained for proteins with a crucial role in sperm capacitation, as the heat shock protein 1-like, 26S proteasome regulatory subunit 7, cAMP-dependent protein kinase type II-alpha regulatory subunit and A-kinase anchoring protein 4. Furthermore, the comparison analysis carried out with IPA showed that some biofunctions have opposite trends in the two comparisons (GO/CTRL T0 and GO/CTRL T2), for instance, infertility, fertility, transport of molecule, metabolism of carbohydrate, infection of embryonic cell lines. To our knowledge, this is the first study comparing protein levels in sperm capacitated with or without GO using proteomics approach. The differences observed among the study groups suggest that the significant changes occurring in the sperm proteome during capacitation in the presence of GO might be one of the contributing factors to the increased fertilizing ability of spermatozoa.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

An optimized method for epigenetic histone 3 modifications (H3K27me3 and H3K27ac) immunodetection in pig spermatozoa

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Improving the selection of boars before entering artificial insemination programs is a priority for breeders, since subfertile boars generate economic losses. Sperm chromatin assessment could be a decisive tool for boar selection. Most of sperm histones are replaced by protamines during spermatogenesis, except for a subset of them demonstrated to have critical epigenetic roles and influence fertilization and gene expression in the early embryo. The acetylated and trimethylated forms of histone 3 (H3K27ac/H3K27me3) are related to DNA transcription activation and repression, respectively, being potential epigenetic targets. Our objective was to optimize the immunostaining detection of H3K27ac and H3K27me3 and determining their location in the boar spermatozoon.

Frozen sperms samples in straws (3 per boar), of 3 boars (20-24 months of age) of Large White breed with a fertility and sperm quality proved, were thawed (37 °C, 30 s), centrifuged and fixed in 4% paraformaldehyde (PFA). The samples were washed and resuspended in PBS, spread on slides and dried. After 20 min, samples were permeabilized for 30 min with Triton X-100 (0.1% or 0.5%) in PBS, washed and decondensed with dithiothreitol (DTT) 10 mM, 20 mM, or 25 mM (15 min, 37 °C), then blocked with 3% BSA for 1,5 h. Primary antibodies (Abcam) were tested at 1/100, 1/200, 1/500, or 1/1000 for H3K27ac (ab177178) and 1/100 or 1/200 for H3K27me3 (ab6002), incubating overnight at 4 °C. After washing, the slides were incubated with the secondary antibody (Abcam;1/400: ab150081 for H3K27ac and ab150120 for H3K27me3) for 2 h, washed and mounted. In addition, negative controls using only secondary anbibody were used. Samples were assessed by fluorescence microscopy, using ImageJ v. 1.54 to estimate fluorescence intensity at least in 200 spermatozoa per slide. The experiment was triplicated using different males, and statistical analyses were performed using the R statistical software and ANOVA. The best protocol was then used to analyze the fluorescence localization patterns.

The optimal fluorescence intensity and definition were obtained using Triton X-100 0.1% and DTT 20 mM at 1/100 both primary antibodies: 69330,9±11315,8 UA for H3K27me3 ($p<0,01$ respect the rest of treatments) and 104298,7±4520,8 UA for H3K27ac ($p<0,001$ respect the rest of treatments). H3K27ac signal varied between boars and was mainly located on the acrosomal region in two boars (>50% of sperm) and on the postacrosomal region in one boar (>90% of sperm). However, H3K27me3 signal appears as a crown around the sperm head or almost throuhout the periphery of the sperm head in all boars (>70% of sperm taking into account both signals).

Future research will aim at testing if these epigenetic marks are related to chromatin status and boar fertility. The main interest is contributing to the early detection of subfertile boars, reducing the subsequent economic losses.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Bull spermatozoa tolerance to natural extracts from grape marc with antimicrobial properties

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The cattle breeding industry uses semen extenders containing wide-spectra antibiotics, which are of concern because of the risk of contributing to antimicrobial resistance (AMR). Polyphenol-rich plant extracts have been tested as supplements in semen extenders because of their antioxidant properties, but their antimicrobial effects have been little explored. Sustainable and environmentally friendly methods allow the revalorization of grape marc as agricultural waste, obtaining extracts with antimicrobial properties (doi: 10.1007/s11356-019-07472-1). We tested the tolerance of bull spermatozoa to the extender supplementation with two grape marc extracts (Pex and Eex differing on solvent extraction, proprietary from i-Grape, Spain). Ejaculates (artificial vagina) from five adult Holstein bulls were pooled, extended in OPTIXcell (IMV), and supplemented with 5 to 30 µl of extract per ml sample. Sperm motility was assessed after 30 min at 37 °C after supplementation (D0) and after 24 h at 5 °C (D1). Motility analysis was carried out with a Nikon E600 microscope (×10 negative phase contrast, Basler acA1920-155uc at 200 fps), processing 1-s videos with OpenCASA v. 2 (https://github.com/calquezar/OpenCASA). The experiment was triplicated, and data were analyzed using linear mixed-effect models (control with no extract as reference).

Eex 30 µl/ml and 20 µl/ml (D0 and D1, respectively) and Pex 20–30 µl/ml showed a negative significant effect in total motility. However, Eex (10–30 µl/ml) and Pex (only at 30 µl/ml) decreased progressive motility in D0, but at D1, only the Pex effect remained. The kinematic parameters were little affected and always showed minor effects. Velocity (VCL, VAP, VSL) significantly increased in D0 with Pex 20 µl/ml and in D1 with Eex 10 µl/ml; linearity (LIN) decreasing in D0 with 30 µl/ml, with no significant effects in D1; head beat cross frequency (BCF) increasing in D0 with Pex 20 µl/ml; and the fractal dimension increasing (hyperactivated-like movement) in D0 for Eex 30 µl/ml and P 20–30 µl/ml, and only Eex 30 µl/ml in D1. No significant effects were detected for other kinematic parameters (STR, WOB, ALH).

Considering the overall motility evaluation, 5–10 µl/ml formulations could be used to extend bull semen. Our findings open the possibility of replacing or reducing antibiotics in bull semen extenders using these sustainably obtained natural extracts, contributing to the circular economy and fighting AMR. Subsequent steps will refine this range for cryopreservation, including antimicrobial efficiency in practical conditions.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Could Estrogen and Progesterone be used as biomarkers in swine saliva?

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Estradiol (17-beta-estradiol, E_2) and progesterone (P_4) play a key role in the regulation of the estrous cycle, and their determination is performed in blood plasma (BP) or serum. Blood collection in pigs is stressful for the animals and requires immobilization for an optimal collection. Saliva is also useful for monitoring ovulation, assessing ovarian function, or testing for pregnancy. Furthermore, its collection is more convenient and less invasive for frequent sampling. Despite these advantages, studies on sex hormones in swine saliva are very scarce, which limits their practical application. In the present study, the levels of E_2 and P_4 in saliva and BP were obtained to determine the potential association between them, and to verify if saliva could be used to monitor the reproductive status in sows. Twenty-seven hormonally synchronized ($PgF_{2\alpha}$, eCG and hCG) sows were sampled 24 hours after the onset of estrus and artificially inseminated. For saliva sampling, sows chewed a zip tied sponge for 15 to 30 seconds; then the sponge was removed from the zip tie and placed in a tube designed for human saliva collection (Salivette®). In the laboratory, the saliva was centrifuged at 1000 rpm for 5 min and stored in 1.5 ml cryovials at -80ºC until analysis. Blood sample was collected from the external jugular vein under general anesthesia. Hormone concentrations in saliva and BP were determined using a solid-phase, enzyme-labeled competitive chemiluminescent enzyme immunoassay (Immulite 1000; Siemens Healthineers). E2 and P4 were detected in all plasma samples, but P4 was only detected in 12/27 (44.4%) saliva samples and 100% of plasma samples (minimum detection level >0.20 ng/ml). P4 levels were higher in plasma than in saliva (8.67±1.94 vs. 1.16±0.74 ng/ml, Wilcoxon non-parametric test, p=0.004), while E2 levels (54.09±6.52 vs. 78.73±8.24 pg/ml, p=0.003) and E2/P4 ratio (25.71±9.76 vs. 251.31±48.39, p=0.002) were higher in saliva than in plasma. The use of Bland-Altman plot to compare plasma and saliva values for E2, P4 and ratio E2/P4 confirmed the inconsistency of the results. This fact confirms that saliva is not a suitable fluid to determine P4 and E2 in pigs with this methodology, a fact that was later confirmed when saliva and BP were obtained from 2 additional pregnant sows (> 60 days of gestation), obtaining P4 values in saliva with a very low value (2.11 ng/ml) when in plasma was 23.38 ng/ml, while E2 was higher in saliva than in plasma (86.1 vs. 15.70 pg/ml). Further studies are needed to clarify the inconsistency of these results in order to use a less invasive technique such as saliva sampling to determine reproductive hormones and assess the reproductive status of gilts and sows.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

Effects of curcumin extract on bull semen samples in in vitro fertilization

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Curcumin extract has demonstrated antioxidant and antibacterial properties at the concentration of 5% in bull semen samples suggesting that it could be used as an alternative to antibiotic[1]; however, fertilization outcomes remain largely unknown. This study aimed to assess the impact of treating bull semen with curcumin extract on *in vitro* fertilization (IVF) results. Bovine ovaries were collected from the abattoir, reaching the IVF laboratory within 4 hours. Upon arrival, cumulus oocytes complexes (COCs) were aspirated and COCs of good grades were selected and randomly distributed between treatment groups, and subsequently matured. All media used was from Stroebech Media ApS, Hundested, Denmark and procedures except *in vitro* fertilization were according to the manufacturer´s protocol. The COCs were matured for 22 hours before fertilization. In total, five batches of ovaries were used, each divided in two replicates containing an average of 30 oocytes. Fertilisation was done with an equal number of untreated control replicates present. Semen samples from one bull with verified *in vivo* and *in vitro* fertility, was diluted to a concentration of 0.5 x 10^6 spermatozoa per mL and treated with curcumin extract at a concentration of 5% for 30 minutes. A lower spermatozoa concentration than recommended was used for IVF, aiming to assess the curcumin effect. Cleavage was assessed 44 hours after onset of fertilization. The parameters that were evaluated were total number of COCs in maturation (OMAT), percentage of cleaved oocytes (CLEAV), percentage of oocytes with more than 2 cells from cleaved (ABOV2) and percentage of blastocysts on day 8 from cleaved (D8). The results (Mean and standard deviation; SD) were as follows for treated and control group respectively: OMAT (311 ± 2.55 vs 317 ± 1.79), CLEAV (50.25 ± 10.89 vs 51.97 ± 13.58), ABOV2 (50.94 ± 19.46 vs 41.88 ± 22.18) and D8 (19.87 ± 15.77 vs 18.23 ± 7.80). In conclusion, sperm treated with curcumin can successfully fertilize oocytes, promote further cell divsions, and had blastocyst formation in IVF.

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Reference

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PHYSIOLOGY AND REPRODUCTION IN MALES AND SEMEN TECHNOLOGY

A longitudinal study of changes in body condition score, testicular morphometry and testicular tissue tone in Sarda rams under traditional management system

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Rams play a crucial role in commercial flocks. During the breeding season, a fertile adult ram can serve at least 5 ewes per day. However, ram infertility is one of the major problems, which is not always given the attention it deserves. In the breeding season of the Sarda dairy sheep (May-November), rams are exposed to high environmental temperatures, and they are often sidelined until the following mating season without the proper care. We can obtain important information on the rams' reproductive condition through the recurrent reproductive evaluations of the ram, including scrotal ultrasonography. The specific aim of this study was to examine the body conformation and testicular parameters of the Sarda rams throughout the breeding and non-breeding seasons. As an initial approach, we recorded the body condition score (BCS), testicular width and tone from March to December. Body condition scoring assessed the muscling and subcutaneous fat development on a scale from 1 to 5 [1-emaciated, 2-thin, 3-average, 4-fat, and 5-obese] and testicular tone was evaluated by palpation using a 3-point scale [1-soft, 2-intermediate, and 3-rigid]. Twenty-eight Sarda rams (2-6 years of age) from 4 commercial farms located in the province of Sassari, Sardinia (3-14 rams per farm) were examined at approximately -2, 2, 4, 6 and 7 month from the introduction to a flock (TFI-between late April and early june); all rams remained in the flock untill December. All rams received 3 s.c., slow-release melatonin implants (18 mg; MELOVINE® Ceva Salute Animale SPA) 20 days before TFI to advance mating and increase their fertility rate. The male/female ratio ranged from 1/40 to 1/50. Preliminary statistical analyses utilized R software to determine the main effects of consecutive examination days (or TFI), ram age, and the interaction of these terms using two-way repeated measures analysis of variance (ANOVA) and Holm-Sidak post-ANOVA test. $P \leq 0.05$ was considered statistically significant. There was a significant main effect of TFI and ram age for the three parameters studied. Mean BCS declined ($P<0.05$) from 2 to 6 months after TFI (3.3±0.09 vs. 2.9±0.05; mean SEM), and it was significantly greater in the 6-year-old compared with the 2-year-old Sarda rams(3.4±0.1 vs. 2.9±0.05). Mean testicular tone was greater ($P<0.05$) 2 mo before TFI (2.4±0.1) compared with 2 (1.6±0.1) and 4 mo (1.8±0.1) after TFI. Lastly, the mean testicular width was greater ($P<0.05$) 2 mo before (5.2±0.09 cm) than 4 mo after TFI (4.8±0.1 cm), and it was greater ($P<0.05$) in 4-year-old (5.3±0.1 cm) compared with 2-year-old rams(4.9±0.08 cm). Our present observations indicate that both the breeding activity and age of Sarda rams can affect their BCS and testicular width, with the most significant declines in those variables occurring by 4 mo (testicular parameters) or from 2 to 4 mo after ram introduction to a herd (BCS), and a rise in testicular size and BCS by 4 and 6 years of age, respectively.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Embryo-induced alterations in the protein profile of bovine uterine extracellular vesicles in vitro

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Effective embryo-maternal communication, crucial for successful pregnancy, is partly mediated via extracellular vesicles (EVs). This study aimed to use uterine explants as an ex vivo model to investigate the communication between blastocysts and the endometrium via EVs, focusing on identifying changes in EV protein profile resulting from this interaction. For this, five synchronized cyclic heifers were slaughtered 7 days after oestrus. From each heifer, four 8 mm circular endometrial explants were obtained from the anterior portion of the ipsilateral uterine horn and cultured individually in 1 mL of protein-free synthetic oviduct fluid (SOF): two were cultured alone (Expl), and two were co-cultured with 5 in vitro-produced bovine blastocysts (Expl+Emb). Also, a group of 50 in vitro-produced bovine blastocysts were cultured alone (Emb) in 500 µL of SOF. Only day 7 blastocysts of excellent or good quality were used. All groups were cultured for six hours at 5% CO2 in humidified air at 38.5°C, after which conditioned medium (CM) was collected for EV isolation. EVs were isolated using size exclusion chromatography, and characterized by detecting CD63, CD81, and CD44 EV markers using flow cytometry. Proteomic analysis was carried out using nanoLC-MS/MS with spectral counting for protein identification and quantification. EVs from five replicates from each group were analyzed. For qualitative analysis, proteins were considered present if detected in ≥3 replicates and were considered exclusive if detected in ≥3 replicates within one group but not detected in any sample within other groups. Bioinformatic analysis was performed with Metascape and Panther tools. We identified 1501 proteins in the CM-EVs from Expl, 1975 in the CM-EVs from Expl+Emb, and 82 in the CM-EVs from Emb. Of these, 66 proteins were detected in the three experimental groups, 1145 were common to Expl and Expl+Emb, none were common to Expl and Emb, none were common to Expl+Emb and Emb, 2 were unique to Expl, and 1 (uncharacterized) was unique to Emb. Moreover, 50 unique proteins were exclusively present when there was an interaction between the endometrium and the embryo (Expl+Emb). These 50 proteins are related to processes such as embryo development (CXADR, MTHFD1L, THOC5, PTK2, MAP7), regulation of stem cell differentiation (EIF2AK2, NELFB, DHX36), and establishment or maintenance of cell polarity (ARF6, PTK2, MAP7, SH3BP1). Additionally, these proteins can potentially modulate endometrial cells, with PTGES2 involved in the eicosanoid metabolic process, EIF2AK2 participating in the interferon tau (IFNT) signaling pathway, and SCRN1 being an IFNT-dependent gene. Our results indicate that the endometrium is responsive to the presence of blastocysts, as evidenced by alterations in the EV protein composition of spent culture medium. Furthermore, these variations may affect embryo development and the IFNT signaling pathway, suggesting EV involvement in embryo-maternal interaction in the first week of pregnancy. Funded by ES-MICIN PID2019-111641RB-I00 & PRE2020-094452.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Age related decline in ovarian hyaluronan; impact on oocyte maturation and embryo development

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It is known that the fertility of females and the chance of producing viable and developmentally competent embryos decreases with age. Recent reports have shown an age-related decline in ovarian hyaluronan (HA), associated with increased stiffness of the ovary texture due to changes in ovary extracellular matrix components, collagen replacement of HA. Aneuploidy during meiosis is prevalent in aged oocytes and is the prime contributor to decreased gamete quality with age due to incorrect chromosome segregation. We proposed that the HA content of oocytes is crucial for oocyte nuclear maturation and its decline will have negative effects on meiotic progression of the oocyte and mitotic cell division in cleaved embryos.

Using ovaries of young and aged mice (3 months v. 22 months) and dogs (3-4 months v. 5-6 years) we explored how ageing affects collagen and HA levels. Collagen was stained using picrosirius red stain, and HA was stained using Hyaluronan Binding Protein immunohistochemistry.

In dog ovaries, there was a dramatic increase in collagen deposition with age throughout the ovarian stroma (7.34% vs 25.82%, $p<0.0005$). Within primordial follicles, the pre-granulosa cell layer of young dogs had less collagen deposition than in old dogs (0.65% vs 2.18% respectively). Collagen deposition in both the theca and granulosa cell layers increased with age in secondary follicles (theca: 14.59% vs 27.86%, $p=0.0185$; granulosa: 0.37% vs 1.24% $p<0.05$). No difference in collagen deposition within the granulosa or theca cell layer of tertiary follicles between young and old ovaries. Unlike in the dog ovaries, there was a significant increase in collagen deposition in the stroma surrounding the vasculature in ovaries of aged mice (16.95% vs 50.46%, $p< 0.005$). In mouse ovaries, collagen deposition patterns were similar to that found in dog ovaries; all three follicle types, collagen deposition showed an increasing trend with age in the theca cell layer (primary: 0.76% vs 12.51%, secondary: 1.15% vs 7.04%, tertiary: 1.49 vs 36.96% in young and old mice respectively).

Whilst there was no significant difference in HA identification within the stroma between the ages in dog ovaries, there was a decrease in HA present in the blood vessels with increasing age (91.98% vs 15.33%, $p<0.0001$). A higher amount of HA was found in the theca cell layer in the younger dog ovaries (59.57% vs 38.01%, $p=0.08$). Similarly, HA expression decreased with age in the granulosa cell layers of secondary and tertiary follicles (secondary: 17.69% vs 4.07%, $p< 0.05$) (tertiary: 7.39% vs 0.58%, $p< 0.05$ respectively). In mouse ovaries, HA staining increased with age in the granulosa cell layer (15.90% vs 36.86%, $p<0.05$) of secondary follicles, but showed no difference in the theca cell layer (16.65% vs 23.12%) between the ages. However, in tertiary follicles, the amount of HA did not differ in the granulosa cell layer between the ages (2.06% vs 3.67%) yet increased with age in the theca cell layer (10.82% vs 77.46%, $p<0.001$). Finally, blood vessels contained a significantly higher HA amount in younger mice when compared to the older (59.57% vs 21.34%, $p<0.005$).

Further, we carried out in vitro culture of bovine cumulus oocyte complexes in the absence or presence of a HA synthase inhibitor (4-methylumbelloferone; 4-MU). The inhibition of HA synthesis during oocyte maturation reduced nuclear maturation to MII stage ($p<0.05$), and cleavage rate after in vitro fertilisation (49 ± 4.8% v. 76 ± 9.8% in control). Importantly, no blastocyst could be produced from the cleaved oocytes (31 ±4.9% in control). Supplementation of HA to 4-MU treated oocytes during in vitro maturation reversed some of the effects (56 ±6.7%, $p>0.05$). Inhibition of HA synthesis post-cleavage by 4-MU blocked embryo development in a dose-dependent manner ($p<0.5$). Similarly, Inhibition of HA receptors RHAMM and CD44 in post-cleavage embryos, resulted in arrest of embryos at early stages (RHAMM, $p<0.001$), or reduction of blastocyst rate (CD44, 11.5% v 19% in control).

In summary, using these multi-species model we have characterised the effect of ageing on ovarian structure, and the potential impact of reduced HA on oocyte and embryo development. Such information may help advancing development of therapeutic techniques to reduce the pathological effects of ageing on female fertility.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Isolation and characterization of extracellular vesicles of oviductal and uterine fluid of receptive rabbit does

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Extracellular vesicles (EVs) are structures released by various cell types and detected in numerous body fluids. EVs mediate cell-to-cell communication by transferring biomolecules (i.e. mRNAs, miRNAs, proteins) that can modulate the activities of recipient cells. Within the female reproductive system, EVs have been observed in the oviducts and uterus of various species. However, the EVs concentration in the oviductal and uterine fluids of rabbits, an induced ovulatory species, remains to be determined. This study aimed to isolate and characterize the EVs from oviductal and uterine fluid of receptive rabbit does. All rabbit does (n=15 animals) were synchronized with 25 I.U. of equine chorionic gonadotropin (Serigán, Lab. Ovejero, León, Spain) i.m., and after 48 h, 20 µg of gonadorelin i.m. (Cystoreline, Ceva, Spain) was applied to induce ovulation. After at least 14-15 h, all animals were euthanized (with barbituric overdose), and after a laparotomy, ovaries and the rest of the reproductive tract were extracted. Oviducts and uterine horns from each doe were flushed separately with PBS (1.5 mL and 2 mL, respectively) and each sample was subjected to 3 series of centrifugation at 4 °C to discard the pellet, while the resulting supernatant was frozen at -80 °C. Subsequently, EVs from oviducts and uterine horns were isolated by size exclusion chromatography (SEC) and concentrated using an Amicon Ultra-15 filter (Merck-Millipore Ltd., Ireland) to a final volume of 100 µL per sample. Nanotracking (NTA) analysis was conducted to characterize the size and concentration of particles present in the oviductal fluid (OF) and uterine fluid (UF) pools (5 pools of 3 animals/fluid) and morphology was evaluated by transmission electron microscopy (TEM). The EVs concentration and size were evaluated by student´s T test. The NTA results revealed that the concentration of particles was lower ($P<0.05$) in OF (2.74×10^9 particles/mL) compared with UF (6.06×10^9 particles/mL). In addition, the mean size was lower ($P<0.05$) in OF (210.5 nm) compared with UF (232.9 nm) while in modal size no differences were found (169.2 vs 181.7 nm, respectively). Additionally, TEM analysis confirmed the presence and morphology of EVs in oviductal and uterine samples. In addition, EVs in both fluids were positive for EVs proteins (CD9, HSP70 and ALIX), and negative for CANX (negative control). In conclusion, the analysis confirmed the presence of EVs in oviductal and uterine fluids from receptive rabbits, with particle concentrations and sizes consistent with EVs. However, more studies are needed to determine whether the differences found have a specific biological role in reproductive health and the promising potential of EVs as biomarkers for comprehensively understanding and monitoring female fertility.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Intergenerational inheritance of large offspring syndrome in IVP dairy cows

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The number of bovine IVP embryos is increasing worldwide, but ~10% of IVP-derived calves are born too heavy (compared to ~2% with AI or MOET). The reason for this large offspring syndrome (LOS) is not yet clearly defined, but it seems to be more prominent in female IVP calves [1]. In addition, little is known about the inheritance of birth weights among IVP-derived progeny.

This study aimed to monitor the birth weight of IVP dairy calves over multiple generations.

Birth weight data of 9112 IVP calves (Holstein Friesian), comprising up to four generations, was analysed. A LOS calf was defined as being larger than the 97th percentile birth weight of 1081 male and 1329 female AI controls, equivalent to ≥47 kg for females and ≥52 kg for male calves. Both mean birth weight and percentage of LOS animals was calculated. Intergenerational birth weight data were analyzed using a General Linear Mixed Model (GLMM) with normal distribution, applying fixed effects for LOS (mother), sex (calf) and recipient parity (heifer/cow), and random IVF bull effects. The proportion of LOS was analysed by chi-square analysis. Statistical significance was accepted at p<0.05.

We did not observe a cumulative increase in birth weights over four successive generations of IVP animals. The mean birth weights of 18 great-grandmothers, 20 grandmothers, 22 mothers and 49 calves were 39.6 ± 0.9, 42.0 ± 0.8, 41.6 ± 0.8 and 40.4 ± 0.5 kg, respectively (p=0.128 GLMM).

Comparing the last two generations from this data set, we identified 12 LOS and 567 non-LOS mothers with an average birth weight of 50.8 kg and 39.9 kg, respectively. The average birth weight of the IVP offspring from LOS mothers was 42.2 ± 0.6 and 44.3 ± 0.6 kg for females and males, respectively. For non-LOS animals, it was 40.9 ± 0.2 and 43.6 ± 0.2 kg. The difference in birthweight between the LOS male and normal male calves was not significant, but there was a significant difference (p=0.043 GLMM) between the birth weights of LOS female and normal female calves.

A similar pattern was observed when looking at the proportion of LOS calves from both mother groups. For the male calves, the percentage of LOS calves did not differ significantly (6/82=7 vs 81/1564=5% for LOS vs non-LOS mothers, respectively), while there was a significant (p =0.008 Chi-square) increase in female LOS calves (12/70=17% vs 103/1240=8% for LOS vs non-LOS, respectively).

We conclude that successive IVP rounds over at least four generations did not significantly increase calf birth weight. However, LOS mothers produced disproportionately more LOS daughters than non-LOS mothers. This compounding effect was not observed in sons of LOS mothers, which were normal. This intergenerational skewing of LOS inheritance across the maternal lineage may point to epigenetic errors related to incorrect maternal imprinting or X-chromosome inactivation dynamics.

Reference

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Lipotoxicity during bovine in vitro oocyte maturation induces genome-wide DNA hypermethylation in post-hatching day 14 embryos

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Maternal metabolic disorders are associated with elevated free fatty acids in the follicular fluid (FF), predominantly palmitic acid (PA). Mimicking this by adding lipotoxic PA concentrations during bovine in vitro maturation (IVM) can induce persistent DNA methylation alterations in the resulting blastocysts, despite being morphologically normal. After the blastocyst stage, the epigenome undergoes a re-establishment of DNA methylation patterns. Therefore, we aimed to examine if DNA methylation patterns were still altered in day 14 extra-embryonic tissue (EXT) originating from oocytes matured in vitro under elevated PA conditions.

This is a follow-up study of Desmet et al. (2020, Human Reprod. 35:293-307). Bovine cumulus-oocyte complexes (COCs) were in vitro matured (24h) under two conditions: 1) BASAL: physiological concentrations of PA, stearic (SA), and oleic (OA) acid (28, 21, and 23 µM); and 2) High PA (HPA): 150 µM PA, and physiological SA and OA. COCs were in vitro fertilized and cultured in control conditions until day 7. Blastocyst rates were significantly decreased in HIGH PA (4.6% decrease; $P<0.05$). Normal and expanded blastocysts (equal proportions) were transferred to healthy cows (8 blastocysts/cow, 8 cows, 5 replicates). Embryos were recovered at day 14 (n=46) and assessed under a stereomicroscope. HIGH PA significantly reduced the proportion of tubular embryos compared to BASAL ($P<0.05$), but not the length ($P>0.1$). Embryo sex was determined using the amelogenin sequence length polymorphism. For the present study, EXT sections were snap-frozen for Methyl-MiniSeq® (Zymo Research, Irvine, CA, USA) to measure genome-wide DNA methylation patterns. Only male tubular embryos were analysed (6 BASAL and 4 HPA) as they were the most abundant and to minimize variation. Sequence reads were aligned to the bosTau9 genome using Bismark 0.19.0, with a minimum of 5x coverage for considered methylation sites. Data were analyzed using *MethylKit* in R to determine differentially methylated probes (DMPs) with FDR 0.05 and 25% differential methylation cut-off. Genomic features were annotated using the *genomation* package.

When comparing HPA to BASAL, 43,864 differentially methylated probes (DMPs) were counted (on 1,048,574 analysed positions), of which 35,389 were hypermethylated (80.68% of total). This relative hypermethylation was evident in all chromosomes (range: 75.12% - 83.46%). The total number of unique genes (accession numbers) that were differentially methylated (>5 DMPs/gene) in HPA compared to BASAL was 2369.

In conclusion, we show that lipotoxicity only during IVM can induce long-term effects on day 14 EXT DNA methylation patterns despite the epigenetic reprogramming during early embryo development and post-hatching growth in a healthy uterine environment. In general, the majority of the DMPs in the HPA group were hypermethylated compared to BASAL. Since DNA hypermethylation is associated with silencing of gene transcription, this might have functional implications. The previous RNA-sequencing analysis of these samples revealed transcriptomic alterations. We are currently integrating the epigenetic and transcriptomic data and annotating the affected genes.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Follicular fluid extracellular vesicles supplementation during in vitro maturation of bovine cumulus-oocyte complexes: preliminary results for effects on oocyte competence and cumulus cells gene expression.

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In vivo oocytes develop in follicular fluid (FF) containing extracellular vesices (fEVs), which carry bioactive molecules that affect cellular function. The present study aimed to examine effects of fEV during IVM on oocyte (OO) maturation and subsequent embryo development, and their mRNA contents related with lipid metabolism and epigenetic regulation. Expression of the same transcripts in cumulus cells (CC) was also determined. Bovine cumulus oocyte complexes (COC) and FF were separately collected by aspiration of 3-6mm follicles from abattoir ovaries. fEV were isolated by size exclusion chromatography from 1ml FF (SEC, qEV1 columns 35 nm Gen 2, Izon) and ultracentrifugation (100,000 x g for 70 min at 4°C, Beckman Coulter), and transcripts within fEVs analyzed in part of the samples by RT-qPCR. The other part of fEV samples was used for IVM medium supplementation. COC were matured in medium (TCM199 with 0.4 mM glutamine, 0.2 mM pyruvate, 50 mg/mL gentamicin, 20 ng/ml EGF) supplemented with 10% fetal calf serum (FCS, control), 10% FCS depleted of its own EV (dFCS) or 10% dFCS+fEV (fEV), at 38.5°C and 5% CO_2 in air, for 24h. After IVM, CC were removed from a subset of the COC, and denuded OO evaluated for first polar body extrusion (1st PBE) and mitochondrial activity (FI=fluorescence intensity, MitoTracker Orange CMTMRos, Thermo Fischer Scientific), while CC were assessed for lipid metabolism and epigenetic regulation transcripts by RT-PCR. The remaining COC were submitted to IVF and assessed for day 7 (D7) blastocyst rates and mitochondrial activity in embryos. The Kruskal-Wallis test was used to analyze the rates of maturation, blastocyst, and ΔCt values (at least 3 replicates) and Mann-Whitney test for mitochondrial activity; significance was 5%. Maturation rates (1st PBE) were not affected by treatments (75.9 to 78.9%, n=220-222 per group). Mitochondrial activity in oocytes was higher in fEV [28814 (n=22) *vs* 21566 (n=23), and 23397 FI (n=23), for FCS and dFCS, P<0.05]. D7 blastocyst rates were unaffected [~ 40%, n= 347-364 per group, P>0.05), as well as their mitochondrial activity (11232 to 13610, n=12-16 per group, P>0.05). Transcripts for DNMT1, DNMT3A, MAT2A, SHMT2 (epigenetic regulators) and PLIN2, LDLR, CD36 and FABP5 (lipid metabolism) were present in fEV and were also expressed in CC. Transcripts abundance in CC was not affected by treatments (P>0.05). In conclusion, maturation and embryo development were not affected by fEV, but mitochondrial activity in oocytes treated with fEVs during IVM was increased, suggesting they may transfer molecules affecting mitochondrial function. Although lipid metabolism and epigenetic regulators were detected in fEV, their abundance was not changed in treated CC, indicating lack of transfer of studied mRNA from fEV to cells. However, as fEV carry other molecules, effects on other functions cannot be ruled out. As these are preliminary results, analysis of lipid contents and of more replicates for mitochondrial activity are ongoing. Financial support: FAPESP (SPEC Grant # 2021/09886-8; AR Grant #2021/06760-3); FS - DS Scholarship (Capes 88887.694635/2022-00); AB - PD Scholarship (FAPESP 2023/01524-5); JRQO - ScI Scholarship (FAPESP 2023/12424-1); LCZJ DS Scholarship (Capes 88887.836321/2023-00); LCM - ScI Scholarship (PUB-USP 2023/83-1).

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

A single-cell transcriptomic atlas of sheep gastrulation and conceptus elongation

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Gastrulation involves the differentiation of three germ layers (ectoderm, mesoderm and endoderm) from the epiblast. In ungulates, gastrulation occurs in the embryonic disc (ED) during conceptus elongation, upon the formation of the primitive streak and concomitant to the massive proliferation of the extraembryonic membranes. Our aim was to analyse the development of embryonic and extraembryonic lineages during gastrulation and conceptus elongation by single-cell RNA sequencing in the sheep. Using the Evercode™ WT Mini kit (Parse Biosciences), we obtained scRNAseq profiles from 80 embryos collected *in vivo* from superovulated ewes at embryonic days (E) 11 (spherical; n = 15), E11.5 (spherical and ovoid; n = 25), E12.5 (tubular and filamentous; n = 25 isolated EDs + extraembryonic membranes [EEMs]) and E13.5 (filamentous; n = 15 isolated EDs + EEMs), and from 216 day (D) 14 *in vitro* embryos (from 3 IVF replicates), cultured in N2B27 medium supplemented with activin A and ROCK inhibitor from D6/7 (Ramos-Ibeas *et al.*, 2022; Development). Transcriptomes of 21,551 cells passed quality controls, with a median of 4,584 genes detected per cell. Unbiased cluster of all *in vivo* embryos (UMAP) and known cell-type markers allowed us to identify 15 clusters, as well as novel sheep cell-type marker genes. Trophectoderm (TE) cells expressed *DAB2*, *GATA2*, *CDX2, TFAP2A* and *TFAP2C*, among other specific markers. A TE sub-population with high interferon-tau (*TP-1P8*), *PAG11, FURIN* and *PTGS2*, and low *CDX2* and *PTGES* expression, proliferated from E11.5 and showed enriched GO terms in lipid metabolism and transport, and PPAR signalling pathway. All hypoblast cells expressed *GATA4*, *FN1*, *HNF1B* and *APOA1*, while visceral and parietal hypoblast specifically expressed *PRDM1* and *TDGF1*, respectively. Anterior visceral hypoblast (AVH), expressing *CER1*, *NODAL*, *EOMES* and *OTX2*, was identified in E11 and E11.5 embryos. Epiblast cells expressed *SOX2*, *SALL2*, *DNMT3B, GPC4* and *PHC1*, among other markers. *TBXT*, *WNT3* and *CDX1*-positive primitive streak cells emerged separating from the epiblast cluster at E11.5, while anterior primitive streak cells, identified by *GSC*, *CHRD, NODAL* and *CER1*, appeared from E12.5. Mesoderm cells, expressing *BMP4*, *HAND1, COL3A1* and *SNAI2,* were detected from E11.5, and definitive endoderm (DE) cells, expressing *FOXA2*, *PRDM1*, *SOX17, BMP7* and *POU5F1*, appeared at E12.5. Both populations proliferated rapidly from E12.5 to E13.5. Finally, primordial germ cells (PGCs), expressing *POU5F1*, *NANOG*, *KIT*, *SOX17* and *TFAP2C*, emerged at E12.5, and *GABRP*, *TFAP2A*, *GRHL2* and GATA3-positive amnion cells were detected at E13.5. UMAP of D14 *in vitro* embryos separated clear trophectoderm, hypoblast and epiblast clusters, as well as few AVH and mesoderm cells, being more similar to E11.5 than to more advanced *in vivo* embryos. This single-cell molecular map of lineages differentiation during sheep gastrulation and conceptus elongation uncovers the timing of formation and cellular origin of critical structures within the sheep conceptus and provides clues to achieve gastrulation and conceptus elongation *in vitro.*

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

3D-DNA-FISH localization of DUXC genes during bovine early development: a pioneer factor silenced by heterochromatin vicinity after EGA?

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In bovine embryos, the transition from maternal to zygotic transcription occurs during the 8-cell stage. This activation of the embryonic genome (EGA) is divided into two steps: a low-level wave of transcription (minor EGA) occurring just after fertilization followed by a major burst of transcription (major EGA). This initial onset of transcription activation is regulated by pioneer transcription factors (TFs) like *DUX4* (Double homeobox 4 in humans) which activate the transcription of a cascade of other TFs and chromatin modifiers, increasing the chromatin accessibility to transcription machinery, like RNA Pol II (*Vuoristo et al., iScience 25, 104137, 2022*). In post-EGA mouse embryos, the murine ortholog of *DUX4*, *Dux*, localizes close to heterochromatin (either near the nucleolus or the nuclear envelope), which is linked to repression of its transcription, and in 2-cell-like embryonic stem cells (ESCs) *Dux* is sequestered at the nucleolus periphery to maintain its repression (*Xie et al, Genes&Dev, 36:331-347, 2022*). In bovine, our preliminary results demonstrated that the bovine ortholog, *DUXC*, is expressed as early as the 1-cell stage and that *DUXC* knock-down (KD) with RNA interference induces developmental arrest at the 8-cell stage. Our study aims to determine the localization of the *DUXC* locus in bovine embryos before and after EGA compared to heterochromatin, the nucleolus, and the nuclear envelope. Using DNA-FISH, we analyzed the shape (assessment of volume and sphericity) and position of *DUXC* DNA-FISH signal (with a mixture of 13 fluorescent probes covering the *DUXC* locus) in nuclei (n >30) from the 2-cell to the morula stages. We find that before EGA, *DUXC* sequences are less compact and at a higher distance from DAPI-dense regions. After EGA (16-cell and morula stages) *DUXC* loci are located at the periphery of the nucleus and *DUXC* signal is associated with the telomeres. In agreement with the results in murine ESCs, we demonstrate that *DUXC* in bovine early embryos is located at the nuclear periphery after EGA, which is concomitant with its repression. To delve further into the putative involvement of *DUXC* in the activation of repeated element transcription and chromatin remodeling, we will compare RNA-sequencing data between normal and *DUXC* KD embryos during early development (from the 2-cell to 8-cell stages).

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Haptoglobin supplementation to the in vitro culture improves embryo development and quality in bovine

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Haptoglobin (HG) is a protein synthesized in the liver, which binds free hemoglobin to inhibit oxidative activity. Although traditionally associated with inflammation due to its presence during such processes, it is also a regular component of healthy mammalian reproductive tissues and fluids. Its occurrence in reproductive organs appears to be specific to certain cell types or stages of the reproductive cycle, suggesting a potential role for HG in mammalian reproductive events. Notably, in pigs, HG has been used to promote *in vitro* early embryo development (GarcíaVázquez et al., Scie. Rep., 2021). This study aimed to determine the influence of HG on the developmental kinetics and quality of *in vitro*-produced bovine embryos. Presumptive zygotes were cultured in 25 µL of in vitro culture medium alone (Stroebech Media®) (Control: n=516) or supplemented with 5 µg of HG (H5: n=519) during the entire culture period (Day 1-Day 7/D1-D7) or during two developmental periods: D1-D4: from presumptive zygotes to 16-cells stage (depicting HG effect in the oviduct; $H5_{OV}$: n=446); or D4-D7: from 16-cells to blastocyst (BD7) stage (depicting HG effect in the uterus; $H5_{UT}$: n=438). Embryo development was evaluated at 96 hpi and at D7-8, while the quality of BD7 was assessed by i) mitochondrial activity with MitoTracker DeepRed, ii) lipid content by Bodipy 493/503 and iii) differential staining of inner cell mass (ICM) and trophectoderm (TE) by anti-CDX2 antibody (Biogenix, Fremont) and Hoechst (n~20 per group). Data obtained from 5 replicates were analysed using One-way ANOVA. No differences were observed in the proportion of embryos that reached the 16-cells stage at 96 hpi, which ranged from 70.6±0.4 to 71.7±0.5%. Consequently, a similar proportion of embryos with a delayed development (< 16 cells), which ranged from 15.7±0.6 to 17.8±0.6%, was observed. Blastocyst yield at D7 - 8 was significantly higher ($P < 0.001$) for H5 (28.3±0.4 - 32.8±0.6%, respectively) and $H5_{UT}$ (27.4±0.6 - 32.2±0.9%), compared to Control (23.0±0.5 - 27.9±0.6%) and $H5_{OV}$ (23.7±0.6 - 28.3±0.7%). The mitochondrial activity was lower ($P<0.001$) in BD7 from H5 and $H5_{UT}$ groups, compared with Control and $H5_{OV}$ groups. When analyzing the lipid content, we observed that the total area of lipid droplets in BD7 resulting from H5 during the entire culture period (D1-D7) or from D1-D4 ($H5_{OV}$) was significantly reduced ($P<0.001$) compared with the control and $H5_{UT}$ groups. The total cells, TE, and ICM did not exhibit differences among BD7 produced in all groups. In conclusion, this study indicates that blastocyst development was significantly improved when HG was present with remarkable effects on their quality in terms of mitochondrial activity, and lipid content. These findings underscore the important role of HG in modulating critical facets of bovine embryonic development and quality.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Interaction of culture media and oocyte quality affects in vitro embryo production of Egyptian local goats

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The development of embryos produced in vitro is influenced by culture conditions and oocyte quality. Therefore, this research aimed to study the effect of oocyte quality and types of culture media on the embryonic development of goat. According to Wieczorek *et al.,* 2020 the first experiment was done using 920 good COCs retrieved (6 replicates) from slaughterhouse ovaries (a local abattoir in Cairo, Egypt, October, 2023). In vitro maturation (IVM) was done using the standard procedure of goat IVM (Wieczorek et al., 2020; AbdElkhalek et al., 2024). In vitro fertilization (IVF) of matured oocytes was conducted using epididymal spermatozoa. The presumptive zygotes were cultured in three different media known as basic medium (G1), SOF medium (G2), and GT-L™ medium (G3). In addition, the embryonic development was monitored for seven days. In the second experiment, 730 COCs (6 replicates) were divided morphologically into two groups: (G1) good-quality COCs and (G2) low-quality COCs. The COCs were in vitro mature and fertilized, as done in the first experiment. The presumptive zygotes were vitro cultured in the best medium selected from the first experiment. Cumulus expansion score and nuclear maturation rate (GV; GVBD; MI and MII) were evaluated using Hoechst staining (Ghanem *et al.* 2021). In the two experiments, embryonic development was assessed in terms of cleavage, morula and blastocyst formation rates (No. of blastocysts embryo No. of culture oocytes). The quality of produced embryos was evaluated by estimating the total cell number using Hoechst staining. Moreover, samples (embryo and oocytes) were used for measuring cytoplasmic mitochondrial activity and lipid content by applying staining of Mito-Tracker green and Nile red stains, respectively. Data of this study indicated that the embryonic development rate (cleavage and blastocyst rates) was higher in the group cultured with G-TL™ (50.56 and 42.83% respectively) than in basic (5.96 and 2.64% respectively) and SOF media (23.1 and 12.25% respectively). G1 had the lowest ($p \leq 0.01$) mitochondrial fluorescent intensity, lipid fluorescent intensity, and total number of embryonic cells, whereas G3 had the highest intensities (59.25, 18.73, and 32.63 vs. 106.1, 45.33, and 62.86, A.U respectively).In contrast, the G2 group's results for the same parameters were 83.69, 28.93, and 43.13 A.U, respectively. In the second experiment the nuclear maturation rate (extrusion of first polar body (non-invasive assessment; morphology) and Metaphase II % (invasive assessment by Hoechst staining) was significantly increased ($P \leq 0.05$) in good (29.79 and 46.67%) compared to bad COCs (9.33 and 13.33%). A higher proportion ($P \leq 0.01$) of oocytes with diffuse mitochondria distribution (increased level of mitochondrial aggregation around the nucleus (central) indicates oocyte maturation) was observed in good (66.67%) than low-quality COCs (6.64%). In conclusion, selecting good quality oocytes and culturing presumptive zygotes in G-TL TM medium improved goat in vitro embryo production (IVP).

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

NODAL is not required for pre-gastrulation embryo development in sheep

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NODAL signalling is essential for mammalian embryo patterning before and during gastrulation. In mice, *Nodal* is initially expressed at the egg cylinder stage and its ablation impairs the formation of anterior visceral endoderm and mesoderm. In ungulates, *NODAL* expression starts earlier, in the inner cell mass at the blastocyst stage, and is later expressed by the epiblast and visceral hypoblast in the embryonic disc (ED), but its role is unclear due to the lack of a knock-out (KO) model. The objective of this study was to analyse the role of *NODAL* in ovine embryo development through CRISPR-mediated genome editing. *NODAL* KO embryos were generated by introducing a stop codon at the first exon using cytosine base editor (BE3). *In vitro* matured oocytes were microinjected with BE3-encoding mRNA and sgRNA (BE+G, containing KO embryos), or with BE3-encoding mRNA alone as microinjection control (BE, only formed by wild-type -WT- embryos). Microinjected oocytes were fertilized and embryo development was assessed at Day (D) 8 (blastocyst stage, cultured in Stroebech IVC medium) or at D12 (right before gastrulation, following an extended culture in N2B27 medium from D6/7). D8 and D12 embryos were imaged, fixed and immunostained to detect SOX2 (epiblast marker), SOX17 (hypoblast marker), and CDX2 (trophectoderm marker). Embryo genotyping in BE+G group was performed by Sanger sequencing. Embryos were considered as KO when all alleles showed a stop codon in the target region, heterozygous (Hz) when a non-edited allele was present, or WT if all alleles were unmodified. Blastocyst rate was similar between the group containing *NODAL* KO embryos (BE+G) and the control group (BE) (42.2±3.7 vs. 47.2±3.3%; mean ± s.e.m; 4 replicates; t-test, p>0.05). In BE+G group, 12/25 (48%) D8 blastocysts were KO. No significant differences were detected in the number of SOX2+ (16.9±4 vs. 12.2±4.6 vs. 9.8±2), SOX17+ (29.2±5.9 vs. 25.7±6.7 vs. 27.3±9.9), CDX2+ (78.2±13.7 vs. 114.2±17.5 vs. 64.3±22.7) or total cells (133.9±15 vs. 159.6±22.8 vs. 153±24.7) between KO, Hz and WT D8 blastocysts (mean±s.e.m; one-way ANOVA, p>0.05). Embryo survival from D6/7 to D12 *in vitro* was similar between BE+G and BE groups (82.5±3.8 vs. 93.8±3.1%; mean±s.e.m; 3 replicates; t-test, p>0.05). In BE+G group, 46/85 (54.1%) D12 embryos were KO. No differences were detected in embryo area (393.1±35.4 vs. 335±40 vs. 386.6±33 μm^2; mean±s.e.m; one-way ANOVA, p>0.05), in the percentage of embryo surface covered by hypoblast cells (63.6±4.5 vs. 51±5.5 vs. 64.5±4.3%; one-way ANOVA, p>0.05), in the number of embryos showing surviving epiblast cells (26/41 [63.4%] vs. 28/33 [84.8%] vs. 34/55 [61.8%]; Chi-square test, p>0.05), in the number of SOX2+ epiblast cells (21.7±4.9 vs. 27.8±4.2 vs. 20.6±4.6; one-way ANOVA, p>0.05), or in the number of embryos developing an ED (6/26 [23%] vs. 11/28 [39.3%] vs. 8/34 [23.6%]) between KO, HZ and WT embryos at D12. In conclusion, *NODAL* is dispensable for ovine embryo development up to pre-gastrulating stages.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Resilience of preimplantation bovine embryos to the availability of energy substrates

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Exposure to metabolic stress during fetal life increases the susceptibility to metabolic diseases in adulthood. This notion is supported by epidemiological and experimental evidence that led to the theory of Developmental Origins of Health and Disease. The adaptive mechanisms the embryo/fetus puts in place to cope with intra-uterine stressors are defined developmental plasticity and entail the capacity of one genotype to generate several phenotypes in response to different environments. To capture the main molecular events of developmental plasticity, we monitored gene expression of bovine blastocysts exposed in vitro to a mild metabolic challenge. With preliminary experiments we standardized the experimental model to remove serum and fertilize by X-sorted semen, to control sources of variability. Then metabolic challenges were given by varying the content in energetic substrates (pyruvate, lactate, glucose, citrate, amino acids) of the culture medium. Three energetic levels, containing 0.5, 1.0, and 1.5-fold increase in energetic substrates, were selected based on the absence of apparent changes in preimplantation embryo development, evaluated by blastocyst rate, distribution of blastocyst stage (early, expanded, hatch-ed/ing), cell number, and pattern of cell lineage specification (N>3; n>31). Genome-wide analysis conducted on 3 independent replicates of 5 expanded blastocysts per treatment revealed minimal differences in gene expression, likely exposing key regulatory genes whose differential expression allowed the adaptation to the changing metabolic environment. Two distinct expression patterns were observed for these genes: progressive upregulation and progressive downregulation along with the increasing energetic availability. The lack of substantial differences seems in line with the general observation that offspring born after a mild intra-uterine exposure to metabolic stress have normal physiological and biochemical parameters until later in life, while a generalized disruption of gene expression would probably impact embryo/fetal/early post-natal life rather than induce a late onset of the disease. Nevertheless, if metabolic stress experienced during preimplantation development were to commit a late phenotype, some kind of mark shall be established at this stage. A possible answer to this question came from the analysis of transcript isoforms. Using a specific bioinformatic pipeline, the presence of two or more transcript isoforms of genes related to epigenetic changes and nuclear reprogramming were detected, indicating that, even in absence of obvious changes in gene expression, the metabolic challenge induced biological effects that can be epigenetically encoded in the embryo. As a proof of concept, acetylation of histone proteins increased when the energetic substrates were higher. These findings shed light on the mechanisms at the onset of developmental plasticity, whereby the activation/repression of few key genes and usage of transcript isoforms confer resilience to metabolic stressors and provide a direct link between changes in the availability of energetic substrates and epigenetic reprogramming.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

High negative energy balance and post-calving period alters the molecular profile of epithelial uterine cells in dairy cows

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Negative energy balance (NEB) during the post-calving period can have a significant economic impact due an overall reduced fertility in dairy cows. This reduction is due, in part, to the impact on uterine biology. The aim of this study was to evaluate if NEB intensity modifies the molecular profile of epithelial uterine cells (EUC) when the uterus is recovering from calving and during the predicted period for starting artificial insemination (AI) in dairy cows, at 30 and 60 days post-calving (DPC), respectively. To characterize NEB intensity, serum samples collected from individual dairy cows at 14 DPC were subjected to metabolite analysis (Low NEB: non-esterified fatty acids, NEFA, 0.3 to 0.8 mmol/L and beta-hydroxybutyrate, BHB, 0.55 to 1.1 mmol/L; High NEB: NEFA ≥ 0.9 mmol/L and BHB ≥ 1.2 mmol/L). At 30 and 60 DPC, the estrous cycles of healthy cows (without clinical or subclinical diseases; 30 DPC n=7, 3 Low and 4 High NEB; 60 DPC n= 6, 3 Low and 3 High NEB) were synchronized using a progesterone-releasing intravaginal device (IVD, 1.9g), estradiol benzoate, and PGF2α analogue. The IVD was removed after 7 days, and estradiol cypionate and PGF2α analogue were administered intramuscularly. On day 9, cows received GnRH analogue and were fixed-time inseminated without oestrous detection. On Day 5 after AI uterine fluid was collected by nonsurgical lavage with 50 ml PBS and EUC were isolated by centrifugation. Total RNA was extracted from EUC and subjected to sequencing analysis. The Nextera XT DNA Library Prep (Illumina) was used to prepare the libraries and sequencing were performed on the NextSeq 2000 (Illumina, USA). Differential gene expression analysis was performed using DESEQ2 R package, considering adjusted P values < 0.10 and an absolute log2 fold change > 0.5. At 30 DPC, we identified 7 genes exclusive to Low NEB, 4 genes exclusive to High NEB and 73 DEGs between the groups, with 24 more expressed in Low NEB and 49 more expressed in High NEB group. At 60 DPC, we found 3 genes exclusive to Low NEB, 8 exclusive to High NEB and 24 DEGs, with 15 more expressed in Low NEB and 9 more expressed in High NEB. Functional enrichment analysis of genes that were exclusive or upregulated in the EUC demonstrated that biological processes are modulated differently in these cells. Genes related to cell proliferation and differentiation were upregulated in EUC from Low NEB cows, while genes related to inflammation and metabolism were increased in EUC from High NEB cows at 30 DPC. At 60 DPC, genes related to immune response, lipid accumulation and conceptus development were upregulated in EUC cells from dairy cows in Low NEB. In contrast, in the High NEB group, genes related to metabolism and cell proliferation were more expressed, suggesting the uterine cells are recovering from this metabolic insult and still present an environment compromised to supporting embryo growth and development at 60 DPC. In conclusion, using RNA-seq approach and dairy cows with different metabolic status during two important periods after calving, this work demonstrates that NEB intensity and post-calving period alter gene expression in EUC.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Evidence that undernutrition in early gestation reduces maternal leptin and impairs AMH in juvenile offspring in dairy cattle

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In beef heifers, undernutrition up to 110 days of gestation (DG) increased maternal testosterone (T) peripheral concentration and diminished the number of healthy growing ovarian follicles in female offspring, as assessed by reduced total number of antral follicles and peripheral concentration of anti-Mullerian hormone (AMH). Leptin (L) production by adipose tissue decreases during weight loss and L can alter androgen production. We hypothesized that maternal nutritional restriction from shortly before conception to two different stages of early gestation would cause 1) an increase in maternal L and T peripheral concentrations and 2) a decrease in AMH circulating concentration in dairy female calves.

Holstein-Friesian heifers (n=42) homogenous for age (14-17mo) and weight (366±41kg) were randomly assigned to three experimental groups and, starting 10d before artificial insemination (AI), were individually fed at: (i) 0.6 of their maintenance energy requirements (M) up to 80DG (Nutrient Restricted, 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. Pregnancy was diagnosed and confirmed via ultrasound 28 and 55DG, respectively. After the end of the differential diet, heifers were group fed ad libitum until calving. Peripheral maternal L and T concentrations were measured in heifers pregnant with a single female calf (NR80, n=8; NR120, n=9; C, n=5). Twenty-two single female calves were born (NR80, n=8; NR120, n=9; C, n=5) and peripheral AMH concentration were measured regularly from birth to 120 days of age (d). Data normality was tested with the Shapiro-Wilk test.Hormonal concentrations were analyzed as repeated measures within treatments using the multivariable linear regression model.

Maternal L concentration was influenced by diet ($p<0.001$) and DG ($p<0.05$), but their interaction tended to be significant ($p=0.075$). Leptin concentration was similar among groups before the start of the differential diet, it was lower in both NR80 and NR120 vs C from 30 to 120DG ($p<0.05$) and was similar among groups from 150DG to calving. Peripheral T concentration in pregnant dams increased as gestation progressed ($p<0.001$) but was not affected by diet. Circulating AMH concentration in female calves was influenced by maternal diet ($p<0.001$) and decreased as calves grew older ($p<0.001$) but was not conditioned by the interaction of maternal diet and age. NR80 and NR120 calves had lower AMH than C from birth to 60d, whereas no difference was detected among groups when calves were 90 and 120d.

In conclusion, maternal undernutrition from preconception to either 80 or 120DG reduced peripheral AMH in female progeny in dairy calves, indicating a potential impairment of ovarian reserve. In the dams, nutritional restriction reduced peripheral L, yet did not influence T concentrations.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Nanoplastic exposure during bovine oocyte maturation affects mitochondrial and developmental process pathways and delays embryo development

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The ubiquity of micro- and nanoplastics (MNPs) has recently gained increasing attention due to their potential effects on health and reproduction. MNPs have been observed to pass biological barriers, including the reproductive tract in rodents, and are able to enter the ovary (Liu, Z. J Hazard Mater. 2022;424(Pt C):127629.) To unravel the effects of nanoplastics (NP; < 1 µm) on oocyte developmental competence, we exposed maturing bovine 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.).

Previously, we discovered that during the in vitro maturation (IVM), 50 nm polystyrene (PS) NPs can enter the oocytes and hamper the oocyte nuclear maturation rate at a concentration of 3 µg/mL. To investigate the underlying mechanisms, RNA-seq was performed on RNA isolated from bovine COCs after the 23h IVM in the 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) at 3 µg/mL at 39°C and 5% CO_2 in air, with the coverage of 20 million reads and 6 gigabases per sample, followed by Gene Set Enrichment Analysis (GSEA) using Kyoto Encyclopedia of Genes and Genomes pathway and gene ontology database. Subsequently, to determine whether the formerly demonstrated decreased oocyte nuclear maturation in response to NP exposure has a sustained effect on embryo development, the day 7 and 8 blastocyst rates were scored after standard IVF and embryo culture in synthetic oviductal fluid with 0.1% BSA (w/v) at 39°C with 7% O_2 5% CO_2 (298 COCs per treatment in 4 replicates). Generalized Linear Model binomial link-logit followed by Bonferroni adjustment for multiple comparisons was used for statistical analysis. A p-value < 0.05 was considered statistically significant.

Following GSEA, pathways related to mitochondrial functions such as oxidative phosphorylation, and mitochondrion organization were downregulated in 50 nm NP-exposed COCs during IVM. Additionally, the analysis indicated a negative regulation of developmental process. Notably, on day 7 of IVP, a significant decrease (p=0.017) was observed in blastocyst rate in the exposure group (36.2±5.8%) compared to the control group (29.2±4.4%), while the cleavage rates on day 5 and blastocyst rates on day 8 were not significantly different between the two groups.

In summary, exposure to NPs during IVM might affect mitochondrial functions, and appears to result in a delayed early embryo development. Further investigations are ongoing to unravel the role of mitochondrial function in the observed toxicity of NPs.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Novel insights into the bull effect on in vitro embryo production: a proteomics approach

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Variations in in vitro embryo production (IVP) depend not only on the quality of the oocytes but also on the sperm used for fertilization. Penetration of multiple spermatozoa into the oocyte, or polyspermy, might be one of the factors contributing to the variation of in vitro fertilization efficiency. This study examines the role of the bull in the process of IVP, from fertilization to embryo development and quality. To do so, bovine cumulus-oocyte complexes were collected from slaughterhouse-derived ovaries, and routine IVP was performed (Wydooghe et al., 2014, Reprod. Fertil. Dev.; 26) with frozen-thawed semen from four bulls. Polyspermy rates from each bull were examined in 13 replicates as previously described (Fernández-Montoro et al. 2024, Reprod. biol. 24, 2). On day 8, embryos at similar stages were collected individually to perform proteomics analyses (n = 16 in high; 23 in low). Generalized mixed-effects models were used to evaluate fertilization and developmental parameters, while MSqRob2 was used for differential expression analysis (FDR of 0.05 and 1.3 $\log_2$ by Benjamini-Hochberg technique). One bull averaged 57% polyspermy rates (high), whereas the other three averaged 20% (low). Although the cleavage and day 7 blastocyst rates were higher in the high polyspermy bull (87 ± 2.5%, $p < 0.01$ and 37.7 ± 3.3%, $p = 0.01$, respectively) compared to the low polyspermy group (76.4 ± 2.9 and 27.4 ± 2.3%), the day 8 blastocyst rate did not show differences among the bull with high polyspermy (41.7 ± 3.4%, $p = 0.26$) and the low polyspermy bulls (37.9 ± 2.5%). Also, no significant differences ($p > 0.05$) were found between bulls for the rate of early, normal, expanded, hatching, or hatched day 8 embryos. Proteome analysis of the blastocysts using label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) allowed the quantification of 6878 proteins. Quantitative analysis showed a total of 942 differentially expressed proteins between the high polyspermy bull and the low polyspermy group, from which 84 were up-regulated and 858 down-regulated. Functional analysis indicated an enrichment of pathways related to oxidative phosphorylation, chromatin organization, transcription, translation, and post-translational modification in low polyspermy bulls. No differences were observed in the proteome profile of blastocyst among the low polyspermy bulls. Our results suggest that polyspermic fertilization can be the cause of embryonic arrest in the bull with high polyspermy rates, as it had the highest cleavage but exhibited similar blastocyst rates to the other bulls. Despite still representing a competitive blastocyst rate, embryos from this bull showed a lower abundance of proteins involved in important metabolic processes, suggesting imbalances that could reduce their developmental potential. In conclusion, our findings demonstrate that a bull with high polyspermy rates can produce embryos at satisfactory rates albeit with diminished quality, potentially affecting further development. Although individual specificity might be present, it is imperative to evaluate polyspermy rates when selecting bulls to be introduced into IVP programs.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Periparturient oleic acid supplementation increases oocyte yield in dairy cows

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The periparturient negative energy balance (NEB) in dairy cows, results in bodyfat mobilization and high levels of non-esterified fatty acid (NEFA), which has been related to the observed reduced fertility performance. In particular, saturated NEFAs are associated with lipotoxic effects on cells, including the oocyte. These effects are counteracted by high levels of oleic acid (C18:1) in follicular fluid and cumulus cells and protect the oocyte against saturated NEFA (Aardema et al., Biol of Reprod 96:982-992,2017). Oocytes that are presumed to be fertilized after a voluntary waiting period of on average 60-100 days postpartum, start their development during the NEB and may be hampered in quality. Around the periparturient period, follicles are exposed to high levels of saturated palmitic (C16:0) and stearic acid (C18:0) via blood (Aardema et al., Biol of Reprod 88:164, 2013). The current study investigated whether periparturient C18:1 fat supplementation counteracts the potential negative effects of NEB on oocytes during the over 120-day follicular growth phase, based on the Britt hypothesis (Britt et al., Bov Proc 24:39-43, 1992).

Pregnant HF heifers (age two years), were pseudo-at-random divided based on BCS and received a fat supplement rich in either C16:0 (78.8%-PA, n=5), which is the standard supplement given in The Netherlands, or rumen-protected C18:1-rich supplement (62,9%-OA, n=6) from 4 weeks before until 4 weeks after calving. Serum samples of -4 weeks (t=0), -2, +2, and +6 weeks post-calving (pc), were analyzed for fatty acids in triacylglyceride (TAG) by HPLC-Mass Spectrometry. At days 50-60, 80-90, and 120-130 pc COCs were two times, with a 5-day interval, collected from 3-12 mm antral follicles by transvaginal ovum-pick-up (OPU). Collected COCs were *in vitro* matured, followed by IVF and embryo culture until day 8, according to our standard protocol. Number of oocytes was analyzed with a Poisson distribution and developmental competence with a logistic regression model, Akaike's information criterion was used for model reduction, to calculate 95% profile (log) likelihood confidence intervals. The study was approved by the ethical committee.

The fatty acid composition in blood of control heifers was dominated by C16:0 at -2 (nearly 60% of total) and + 2 weeks (45% of total), around 30% higher than in the OA group. In the OA group, the C18:1 in blood (25% of total), was 10% higher than in the PA group. At +6 weeks fatty acid compositions were comparable in the groups. In the OA group, a higher number of follicles (21.9±7.9 vs 14.5±6.0) and COCs (13.1±6.8 vs 8.2±4.0) was recorded compared to the PA group. In the OA group, the number of the collected COCs was overall 1.6 times higher in comparison to the PA group. Developmental competence of oocytes was not different between the groups.

Fat supplementation resulted in a change in the fatty acid composition in blood, the OA condition demonstrated a profile richer in C18:1 and lower in C16:0 in comparison to the PA group. Interestingly, OA supplementation resulted in a significantly higher amount of collected oocytes at OPU. These data suggest that periparturient OA supplementation may increase oocyte yield during periparturient NEB.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Isolation and characterization of extracellular vesicles from sheep reproductive fluids

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Extracellular vesicles (EVs) are nanoparticles involved in cellular communication releasing miRNAs, proteins and lipids into target cells modulating cellular response, and are present in biological fluids. Recent works have noticed the role of the EVs from female reproductive fluids in guiding sperm towards the fertilization site and promoting capacitation signaling pathways. The aim of this study was the isolation and characterization of ovine EVs from follicular, oviductal and uterine fluids, comparing two isolation methods (ultracentrifugation (UC) and size exclusion chromatography (SEC)) and fractions (pellet and supernatants). For this end, reproductive fluids from six sheep were pooled after flushing extraction, with each pool comprising samples from two individuals. Concentration, population, and size were evaluated by nanoparticle tracking analysis, and the detection of EVs markers as the CD9, CD81, and CD63 tetraspanins by flow cytometry allowed for their identification. Commercial exosomes expressing green fluorescent protein and CFSE-labelling were used for intact EVs gating in flow cytometry evaluations. Differences between samples were evaluated by one-way ANOVA analysis and Bonferroni test was used for multiple comparations. Our results showed no significant differences ($p > 0.05$) in EVs concentration between fluids. However, the percentage of EVs positive for CD63 was higher ($p < 0.05$) in follicular fluid compared to oviductal and uterine fluids (67.40 ± 1.39 *vs* 62.11 ± 1.33 and 61.58 ± 1.33, respectively). Significative differences ($p < 0.05$) were found in the percentage of CD9-positive EVs present in follicular fluid compared to that in uterine fluid (26.53 ± 1.05 *vs* 22.46 ± 1.00), while no differences ($p > 0.05$) between fluids for CD81 were found. Regarding the isolation method, the EVs concentration obtained was higher ($p < 0.05$) by UC than by SEC ($7.27x10^8 \pm 6.21x10^7$ *vs* $5.09x10^8 \pm 6.21x10^7$ EV/mL). Moreover, the percentage of CD63-positive EVs obtained by UC was higher ($p < 0.05$) than that by SEC (65.38 ± 1.12 *vs* 62.01 ± 1.08), with no differences ($p > 0.05$) for CD9 and CD81 positive populations. Regarding fractions, there were no differences ($p > 0.05$) in concentration, but CD63 positivity was significantly higher ($p < 0.05$) in the pellet than in the supernatant (66.60 ± 1.22 *vs* 60.79 ± 1.02). No differences ($p > 0.05$) were found in CD9 and CD81 populations. Our findings confirmed the superior efficiency of UC method for EVs isolation, revealing differences in the tetraspanin composition of EVs, compared to those obtained by SEC, as well as among fractions. Moreover, we demonstrated the differential EVs populations among female reproductive fluids suggesting specific roles on both oocyte and sperm physiology. Further investigation into the effects of these distinct EVs populations on oocyte competence, sperm fertilization ability and embryo development will provide valuable insights.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Exploring mitophagy dynamics in bovine oocytes during in vitro maturation in response to mitochondrial membrane uncoupling

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Oocytes mitochondria have unique dynamics, morphology, and reactive oxygen species (ROS) production compared to somatic cells, potentially leading to mitochondrial quality control mechanisms that may differ from the typical activation of mitophagy in response to mitochondrial dysfunction. Previous research in inbred C57BL/6 mice has shown that oocytes do not induce mitophagy in response to increased oocyte dysfunction (Boudoures *et al*. 2017, Dev Biol. 426(1):126-138). However, since significant differences in mitochondrial functions in oocytes between outbred and inbred models have been reported, here we aimed to explore the ability of bovine oocytes to induce mitophagy as a response to mitochondrial membrane uncoupling by adding carbonyl cyanide m-chlorophenyl hydrazone (CCCP) during in vitro maturation (IVM).

Cumulus oocyte complexes (COCs) were aspirated from bovine ovaries collected from a local slaughterhouse. Three replicates were conducted with COCs assigned to five experimental groups (80 COCs per group): control (CTR), solvent control (SC) with 0.05% v:v DMSO, BafilomycinA1 (50nM) (BafA1), CCCP (CP)(10µM), and CCCP+BafA1. BafA1 inhibits autophagy by preventing lysosomal acidification, resulting in autophagosome accumulation. After a 2-hour exposure to CTR or CCCP-containing medium, with or without BafA1, COCs were matured for 22 hours. Finally, denuded mature oocytes were used for live-cell staining (JC-1 and CellROX Red) and Western Blotting (PINK1 and LC3 expression levels). Additionally, pools of 50 oocytes of the CTR, SC and CP groups were fertilized and routinely group cultured for 7 days to evaluate developmental capacity.

JC-1 staining confirmed the mitochondrial membrane uncoupling effect of CCCP treatment compared to CTR (P=0.02), accompanied by a decrease in ROS production measured by CellROX signal intensity for CP vs CTR (P=0.001), which may be due to a reduced proton gradient and electron transport. Cleaved (ΔN-PINK1) and full-length PINK1 (FL-PINK1) were quantified using Western Blotting, with the ΔN-PINK1/FL-PINK1 ratio being a measure of PINK1 cleavage, while FL-PINK1 stabilization in dysfunctional mitochondria may indicate activation of the PINK1-PARKIN pathway. Interestingly, CCCP treatment did not lead to lower PINK1 cleavage rates (0.76±0.52) compared to CTR (1.82±1.69) and SC (1.01±0.65)(P=0.62). Comparing the presence of autophagosomal protein LC3 between CCCP versus CCCP+BafA1 and SC versus BafA1, representative for CCCP-induced and DMSO-induced autophagy, revealed that CCCP did not significantly increase autophagy flux compared to DMSO (P=0.93). Remarkably, CCCP-treated oocytes showed similar blastocyst rates to CTR and SC groups after IVP on day 8 (CTR 0.36±0.06, SC 0.34±0.03, CP 0.43±0.01)(P>0.1).

Our findings suggest that bovine oocytes do not activate PINK1/Parkin-mediated mitophagy in response to CCCP-induced mitochondrial uncoupling at the onset of maturation. The reduction in ROS and potential other mechanisms might contribute to normal development to blastocysts. Also, further exploration with additional replicates and examination of other outcome parameters are needed to fully understand the complexities of mitophagy induction in oocytes.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

The impact of a maternal obesogenic diet on offspring's oocyte mitochondrial biogenesis in the primordial follicles at birth and at weaning. Insights from an outbred mouse model

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Maternal obesity presents a global health problem, potentially impacting offspring fertility. Research showed that offspring born to obese mothers exhibit mitochondrial dysfunction in the ovulated oocytes at adult age, including markers of mitochondrial biogenesis. However, it remains unknown if these alterations are inborn. Also, lactation from an obese mother can alter postnatal metabolic programming and may impact offspring oocyte quality. Understanding the timings of mitochondrial alterations is crucial as maintaining a healthy primordial follicle pool is vital for sustaining folliculogenesis and guaranteeing oocyte quality upon ovulation. In this study, we aimed to investigate the impact of a maternal obesogenic (OB) diet on offspring's oocyte mitochondrial parameters in primordial follicles, hypothesizing that it affects mitochondrial biogenesis at birth (TP1) and at weaning (TP2).

Female Swiss mice were fed either a control (C)(n=5) or an OB (n=5) diet for 7 weeks and then mated (control males, cross-over design). The females remained on their allocated diets during pregnancy and lactation. Female offspring were sacrificed at TP1 and TP2. Ovaries were fixed in paraformaldehyde. The expression of Mitochondrial Transcription Factor A (TFAM), responsible for mtDNA transcription, and its activator Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) was examined by immunofluorescent staining and confocal microscopy, using one random section/ovary. For PGC-1α, 103 primordial follicles of the C group (TP1:n=55, TP2:n=48) and 100 primordial follicles of the OB group (TP1:n=71, TP2:n=29) were assessed. For TFAM, 80 primordial follicles of the C group (TP1:n=43, TP2:n=37) and 66 primordial follicles of the OB group (TP1:n=34, TP2:n=32) were assessed. Gray scale intensity was quantified in the ooplasm, and in the nucleus for PGC-1α, using three z-positions. First, the main effects of the maternal diet, timepoint (i.e. TP1/TP2) and their interaction were examined using Two-way ANOVA. For PGC-1α, a site effect (i.e. ooplasm/nucleus) was present and Three-way ANOVA was used. If the interaction was significant, treatment effects at each TP were examined by independent student *t*-tests or Mann-Whitney U tests in case of nonparametric testing.

The effect of the maternal OB diet on PGC-1α expression was dependent on the timepoint ($P<0.001$). Primordial oocyte PGC-1α was not altered by maternal OB diet at birth ($P=0.44$) but was increased in both the ooplasm($P=0.04$) and the nucleus ($P=0.046$) at weaning. TFAM was significantly reduced by the maternal OB diet ($P<0.001$) at both timepoints (interaction $P=0.48$).

In conclusion, since PGC-1α was not affected at birth, this indicates no inborn alterations in mitochondrial biogenesis and other PGC-1α-dependent metabolic pathways in oocytes of primordial follicles in offspring born to OB mothers. The effect on TFAM at birth may induce changes in oocyte mtDNA replication that might be adaptive or reparable in nature. Subsequently, nursing from an obese mother induces postnatal changes in PGC-1α which might partially explain the differences in mitochondrial and metabolic functions described in mature F1 oocytes in earlier studies.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

In vitro procedures deregulate the embryonic disc and extraembryonic membranes transcriptome of day 15 elongated bovine embryos

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In vitro embryo production is intensively applied worldwide for cattle production because of several advantages. Indeed, more than 1.1 million in vitro produced (IVP) embryos were transferred in 2022 compared to 360.000 in vivo produced (IVV) embryos (Viana, EmbryoTransfer Newsletter, 41, 20-38, 2022). However, IVP embryos yield around 25% lower pregnancy rates compared with IVV embryos, with most of the losses occurring at ~day 20 of pregnancy (Ealy et al., J Anim Sci, 97, 2555-2568, 2019), probably because of impaired elongation from ~day 13 of pregnancy (Clemente et al., Biol Reprod, 85, 285-95, 2011). This period involves critical events, such as gastrulation and formation of the embryonic disc (ED), as well as the development of the extraembryonic membranes (EEM). Therefore, we hypothesize that in vitro procedures negatively affect the molecular signatures of both embryonic regions. The goal was to quantify the effect of the in vitro procedures on the transcriptome of ED and EMM of the same length day 15 bovine conceptuses compared to in vivo ones. IVP embryos were derived from oocytes aspirated from slaughterhouse ovaries after maturation, fertilization, and culture in serum-free medium until transfer at day 7, while IVV embryos were generated after ovarian super-stimulation and artificial insemination, following standard protocols for both procedures. Animals were flushed at day 15 of gestation to recover the elongated embryos. Sections of the EMM and ED from tubular same-length IVP and IVV embryos (5.9 ± 0.2 vs 5.6 ± 0.2 mm, respectively; n=4 per group) were submitted to paired-end RNAseq. Raw data were aligned to the ARS-UCD1.3 bovine genome, and the processed files were analysed using the DESeq2 package for the R software to determine differentially expressed genes (DEG) at a false discovery rate (FDR) < 0.05. Enriched biological processes (FDR < 0.05) associated with DEG in the EMM or ED between IVP and IVV embryos were determined using the DAVID database. There were 801 and 1516 DEG in the EMM and ED, respectively, between IVP and IVV embryos. The functional analysis showed that DEG more expressed in the EMM of IVP embryos (429) enriched cell migration, while down-regulated genes (372) were associated with transcription and ontological terms involved in chromatin structure, such as core histones, DNA binding and nucleosome. DEG upregulated in the ED of IVP embryos (564) were involved in glycerophospholipid and fatty acid metabolism. Notably, downregulated (or inhibited) DEG (952) enriched anatomical morphogenesis processes and pathways playing critical roles in embryonic development, such as canonical Wnt, bone morphogenetic protein and transforming growth factor beta signalling pathways. In conclusion, these results demonstrated that the in vitro procedure deregulated the molecular signatures of EMM and ED regions during early gastrulation and negatively impacted the developmental process of IVP embryos, even when they were of similar length to the IVV counterparts, which can explain why most IVP conceptuses are lost after this period.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Oleic Acid prevents the detrimental effect of the combination of saturated NEFAs with Lipopolysaccharide on bovine oocyte competence

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In high-producing dairy cows experiencing Negative Energy Balance (NEB) during the transition period, the mobilization of Non-Esterified Fatty Acids (NEFAs) can coincide with post-partum inflammatory conditions such as metritis, endometritis, and mastitis, resulting in the presence of Lipopolysaccharide (LPS) in both the bloodstream and follicular fluid. This cascade of events may compromise the developmental competence of oocytes. It was previously demonstrated that oleic acid counteracts the toxic effects of saturated fatty acids in vitro (Aardema et al., 2011). This study aimed to investigate whether oleic acid (OA) supplementation during in vitro maturation could mitigate the potential adverse effects of both saturated NEFAs and LPS. For this purpose, abattoir-derived bovine cumulus-oocyte-complexes (COCs) were exposed to different combinations of 100μM of stearic acid (SA), 150 μM of palmitic acid (PA), 10 μg/mL of LPS, and 200 μM of oleic acid for 22 hours of maturation and then fertilized (IVF) and cultured until day 8. A total of 1907 COCs (8 replicates) were divided into 6 experimental groups: Control (n=321), SA+PA (n=319), SA+PA+OA (n=316), LPS (n=310), SA+PA+LPS (n=320), SA+PA+LPS+OA (n=321). Cleavage and blastocyst rates were assessed on respectively days 5 and 8 (Day 0 = IVF). Data were analyzed by ANOVA and results were expressed as mean ± SEM. The cleavage rate of oocytes from the SA+PA (59.6 ± 4.5%) and SA+PA+LPS (63.7 ± 5.1%) groups were comparable and both decreased ($P<0.05$) compared to those exposed to SA+PA+OA (77.2 ± 3.0%). There was no difference between the control, LPS, and SA+PA+LPS+OA (respectively, 71.4 ± 2.4, 66.0 ± 4.7, and 70.3 ± 2.0%). Blastocyst rates were reduced ($P<0.01$) in the SA+PA (17.3 ± 3.9%) and SA+PA+LPS (19.6 ± 2.9%) groups compared to the control (29.0±2.3%). This adverse effect was counteracted by the addition of OA with respectively 31.1 ± 2.2% for the SA+PA+OA and 29.4 ± 2.1% for the SA+PA+LPS+OA group. The group of LPS alone was not different from the other groups (23.5 ± 2.7%). These findings demonstrate that the presence of OA during in vitro maturation (IVM) counteracts the toxic effects of saturated NEFAs (SA+PA) and LPS. However, these results also suggest that oocyte competence is mainly affected by high levels of saturated NEFAs, as no additional toxic effect was observed when LPS was included. Interestingly, LPS alone did not result in a significant reduction in the blastocyst rate of exposed oocytes. In conclusion, the enrichment of the IVM medium with OA is effective at protecting bovine oocytes challenged with high levels of saturated NEFAs and LPS, suggesting a potential application in vivo to alleviate the detrimental effects of NEB.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

SOX17 ablation impairs hypoblast formation and reduces trophectoderm proliferation in bovine embryos

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Following blastocyst hatching, cattle embryos are composed by three lineages: the epiblast –which will form the embryonic disc and subsequently the fetus- and two extra-embryonic lineages –the trophectoderm and the hypoblast-. Post-hatching embryo development accounts for most embryonic losses in cattle and is remarkably divergent from that of mice, the only mammal where it has been thoroughly studied by loss-of-function approaches. In mice, post-hatching development relies on the reciprocal signalling between the three lineages, but the role of inter-lineages signalling remains unknown in other mammals. The objective of this work has been to determine the developmental ability of cattle embryos lacking hypoblast. To generate hypoblast-devoid embryos, the hypoblast-specific transcription factor SOX17 was ablated by CRISPR technology. In vitro matured bovine oocytes (n=378, 4 replicates) were divided in two groups: one was microinjected with Cas9-encoding mRNA and a sgRNA against SOX17 (n=276, C+G group, containing KO embryos), and the other was microinjected with Cas9-encoding mRNA alone, serving as microinjection control (n=102, C group, formed by wild-type WT embryos). Microinjected oocytes were fertilized in vitro and developed to Day (D) 12, by conventional culture in SOF medium to D7 followed by culture in N2B27 medium. D12 embryos were fixed and subjected to immunohistochemistry (IHC) to detect the development of specific lineages and genotyped by miSeq. No significant differences were observed in developmental rates between both microinjection groups (Cleavage rate: 82±3.8 vs. 83.6±1.5%; blastocyst rate: 35.0±9.2 vs. 23.9±4.9%; D7 to D12 survival rate: 73±4.6 vs. 70.2±5.4%; for C vs. C+G, mean±s.e.m., t-test $p>0.05$). 34 out of 39 D12 embryos genotyped in C+G group (87%) were edited, and 15 were KO (38.4%). SOX17 expression was not detected by IHC in SOX17 KO embryos, which failed to undergo hypoblast differentiation as evidenced by the lack of an inner cellular layer beneath the CDX2+ trophectoderm and by the absence of FOXA2+ cells. In contrast, WT and edited non-KO embryos developed hypoblast, but while complete hypoblast migration was observed in 12/20 (60%) WT embryos, no edited-non-KO embryo (0/19) achieved complete hypoblast migration. D12 embryo diameter was significantly reduced in SOX17 KO and edited non-KO embryos compared to WT (0.93±0.09 vs. 0.60±0.04 vs. 0.59±0.07 mm, for WT, edited and KO embryos, respectively, ANOVA, $p<0.05$). No significant differences were observed in embryonic disc formation rate (10/20, 5/19 and 5/15, for WT, edited non-KO and KO embryos, respectively). In conclusion, SOX17 is required for hypoblast differentiation and hypoblast devoid embryos show a reduced trophectoderm proliferation by D12.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

Isolation and characterization of extracellular vesicles from bovine granulosa cells under oxidative stress conditions

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Recent studies have demonstrated the potential of extracellular vesicles (EVs) derived from stressed granulosa cells to induce an adaptive response to cellular stress in recipient cells (Gebremedhn et al. Sci Rep, 10, 15824, 2020). This study aimed to isolate and characterize EVs derived from granulosa cells subjected to oxidative stress. Bovine granulosa cells were obtained by aspirating 3-6 mm follicles and cultured in TCM-199 medium + 10% exosome-depleted fetal bovine serum (FBS). Sub-confluent cells were exposed to 5 µM H2O2 for 40 min to induce oxidative stress followed by 24h of cell culture in TCM-199 medium + 10% exosome-depleted FBS. Non treated cells served as Control. Oxidative stress was confirmed by assessment of mitochondrial activity (Mitotracker) and Reactive Oxygen Species (ROS) production (DCFH-DA) assessing fluorescence intensity in arbitrary units. EVs were isolated from spent media by size exclusion chromatography (SEC; Hansa BioMed) and concentrated using an Amicon Ultra-15 filter (Merck-Millipore Ltd., Ireland) to a final volume of 100 µL per sample. Nanotracking (NTA) analysis was conducted to characterize the size and concentration of particles present in Control and Oxidative Stress (OS) groups (4 replicates/treatment) and morphology was evaluated by transmission electron microscopy (TEM). The induction of oxidative stress in granulosa cells was confirmed by a two-tailed student's T test, revealing higher ($P < 0.05$) levels of ROS in the OS group (17.02 ± 0.19) compared to the Control group (12.15 ± 0.09). Additionally, there was a significant ($P < 0.05$) decrease in mitochondrial activity in the OS group (94.83 ± 1.93) compared to the Control group (140.00 ± 2.91). NTA analysis revealed that particle concentration in the Control group was $2.07 \times 10^{11} \pm 1.6 \times 10^{10}$ particles/mL, with a mean size of 237.3 ± 4.9 nm and a modal size of 170.1 ± 8.3 nm. Particle concentration in the OS group was $1.98 \times 10^{11} \pm 2.2 \times 10^{10}$particles/mL, with mean and modal sizes of 223.2 ± 7.0 nm and 160.0 ± 13.4 nm, respectively. The student's T test did not show significant differences among groups in terms of particle concentration, mean and modal sizes among Ccontrol and OS groups. Additionally, TEM analysis confirmed the presence and morphology of isolated EVs. In conclusion, the induction of oxidative stress in granulosa cells leads to increased levels of ROS and reduced mitochondrial activity. Furthermore, our selected methodology demonstrated effectiveness in isolating oxidative stress-induced EVs from bovine granulosa cells, exhibiting particle concentration and size consistent with known EVs characteristics although no differences were observed in terms of EVs concentration, size or morphology between the two experimental groups. The successful isolation and characterization of extracellular vesicles from oxidative stressed granulosa cells establish an effective methodology for further research, specifically in analyzing the cargo of these EVs to investigate their potential to modify oocyte composition.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY

ZFN10 is dispensable for first and second lineage differentiation events in bovine embryos

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The transcription factors regulating first lineage differentiation, which give rise to the trophectoderm and inner cell mass (ICM), have been thoroughly studied in the mouse model but the roles of key regulators such as OCT4, CDX2 or TEAD4 are not conserved in other mammals, including ungulates. Zinc finger (ZNF) proteins are transcription factors that are involved in gene silencing during mouse embryonic stem cell differentiation and one of the family members (ZFN10) is predominantly expressed by the bovine ICM, thereby constituting a plausible candidate to mediate first lineage differentiation in ungulates. The objective of this study has been to elucidate ZNF10 role during bovine early embryogenesis by assessing the developmental ability of *ZFN10* KO embryos generated by introducing a stop codon by base editing technology. *In vitro* matured bovine oocytes (n=379, 4 replicates) were divided in two groups: one was microinjected with cytosine base editor (CBE) encoding mRNA and a sgRNA against *ZNF10* (n=224, C+G group, containing KO embryos), and the other was microinjected with CBE encoding mRNA alone, serving as microinjection control (n=155, C group, formed by wild-type WT embryos). To assess if *ZFN10* embryos were able to complete first and second lineage differentiation events, microinjected oocytes were fertilized *in vitro* and developed to Day (D) 12 by conventional culture in SOF medium to D7 followed by culture in N2B27 medium. D12 embryos were fixed and subjected to immunohistochemistry to detect trophectoderm (CDX2+) and the two lineages derived from the ICM in a second differentiation event: epiblast (SOX2+) and hypoblast (SOX17+). Following image acquisition, embryos from C+G group were genotyped by Sanger. No significant differences were observed in developmental rates between both microinjection groups (Cleavage rate: 70.4±9.8 vs. 77.5±4.4%; blastocyst rate: 18.0±4.0 vs. 23.6±3.9%; D7 to D12 survival rate: 74.5±2.7 vs. 74.4±5.2%; for C vs. C+G, mean±s.e.m., t-test p>0.05). 33 out of 35 D12 embryos genotyped in C+G group (94%) were edited; 5 were heterozygous (Hz), and 28 were KO (80%). *ZNF10* KO embryos were able to develop up to D12 normally. D12 embryo diameter was similar between WT, Hz or KO embryos (1.0±0.07 mm vs. 1.22±0.09 vs. 0.96±0.09 mm, for WT vs. Hz vs. KO, respectively, mean±s.e.m, ANOVA, p>0.05). Embryonic disc formation rate was also unaffected by ZNF10 ablation (11/25 vs. 3/5 vs. 14/28, for WT vs. Hz vs. KO, respectively, Chi-Square, p>0.05), and the number of epiblast (SOX2+) cells in the embryonic disc was similar between the three genotypes (39±7 vs. 57±17 vs. 51±7, for WT vs. Hz vs. KO, respectively, mean±s.e.m, ANOVA, p>0.05). No significant differences were observed either in the rate of complete hypoblast migration between all three genotypes (14/22 vs. 5/5 vs. 21/28, for WT vs. Hz vs. KO, respectively, Chi-Square, p>0.05). In conclusion, ZNF10 is not required for first and second lineage differentiation events.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CLONING, TRANSGENESIS, STEM CELLS

A Decade of health and genomic stability: the cattle produced by transposon-mediated transgenesis and embryo transfer

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In previous study, we produced one fluorescent transgenic female and one fluorescent transgenic male cattle (SNU-SB-1, SNU-PB-1) using transposon system. Although the transgenic animals has led to concerns about their long-term health and genomic stability, there are a few studies. In this study, we assessed these factors in the transgenic cattle over a decade by employing a transposon-mediated transgenesis and embryo transfer technique. Our longitudinal study included health evaluations and complete genome re-sequencing. Using blood analysis, we observed no significant changes in physiological parameters in the cattle, which are now over ten years old. To assess the effect of transposon-mediated transgene insertion on genome stability over a 10 year period, whole-genome DNA sequencing was performed using blood sample from the transgenic cattle and one age-matched wild-type cattle. As a result, in the 10-year-old transgenic cattle, the number of genomic variants detected was comparable to that in wild-type cattle. SNU-SB-1 and SNU-PB-1 contained 6,155 and 7,990 somatic SNPs and 3,367 and 3,652 somatic Indels, respectively. Among these variants, 17 and 9 non-reference homozygous (NonRefHom) SNPs were found in SNU-SB-1 and SNU-PB-1 cattle, respectively, along with 132 and 111 NonRefHom Indels. In somatic structure variants(SVs), six and two somatic SVs were detected in SNU-SB-1 and SNU-PB-1 cattle, respectively, all of which were heterozygous. The copy number variations (CNVs) in 10-year-old transgenic cattle mirrored those detected in 1-year-old transgenic cattle, indicating the absence of somatic copy number alterations over 10 years in transgenic cattle. Taken together, our whole-genome DNA resequencing data indicated that transposon-mediated transgene insertion in transgenic cattle did not perturb genome stability over the course of a decade. These results highlight the feasibility of using transposon systems for creating transgenic livestock, with potential broader applications in agriculture and biotechnology. This research greatly enhances our understanding of the long-term effects of transgenesis in large animals, affirming the safety and stability of the method.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CLONING, TRANSGENESIS, STEM CELLS

Effects of holding immature porcine oocytes on in vitro maturation and parthenogenetic embryo development

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The growing significance of pigs as both animal models and potential organ donors in biomedical and biotechnological research has sparked interest in *in vitro* porcine embryos production. However, challenges persist in achieving oocyte complete developmental competence. Delaying meiotic progression before *in vitro* maturation (IVM) by holding immature oocytes has been shown to have potential benefits in horses, but its effect on porcine oocytes is unclear (Lazzari G., JEVS, 89, 103097, 2020). This study aimed to investigate if pre-IVM holding and modulation of the IVM timings could improve porcine oocyte maturation and parthenogenetic (PGA) embryo development. Swine cumulus-oocyte complexes (COCs, 30/group) were directly subjected to IVM and cultured in TCM199 with 10% fetal calf serum, additives, and hormones under 5% CO2 at 38 °C (a). Alternatively, they were kept in H-SOF in the dark at room temperature for 24 hours (h) before IVM (b: 24hHOLD). For each group 4 timing were considered: (1) a. COCs subjected to 26h-IVM; b. 24hHOLD + 26h-IVM; (2) a. COCs subjected to 42h-IVM; b. 24hHOLD + 42h-IVM; (3) a. COCs subjected to 46h-IVM; b. 24hHOLD + 46h-IVM (4) a. COCs subjected to 50h-IVM; b. 24hHOLD + 50h-IVM. All experiments were conducted in at least 3 biological replicates and the data were analyzed using one-way ANOVA with Tukey's multiple comparison test. Following IVM, cumulus cells expansion was evaluated, COCs were denuded, and maturation was assessed by identifying first polar body extrusion of metaphase II (MII) oocytes. Matured oocytes were PGA activated by a double electric pulse of 1kV/cm in activation medium (0.3M mannitol and 1mM Ca^{++}). Our results revealed that oocytes exposed to 26h-IVM, with or without 24h pre-IVM holding, did not reached the MII stage, as expected. Preliminary data indicated that maturation rates did not differ between the 42h, the 46h and 50h-IVM timings and their respective 24hHOLD groups, ranging from 70.15 ± 4.66% to 79.14 ± 7.16%. By contrast, significantly lower (P≤0.05) cleavage rates were identified for 42h-IVM group (59.85 ± 11.44%) and its 24hHOLD counterpart (52.42 ± 10.94%) compared with 46h (73.77 ± 7.21%) and 50h (86.65 ± 4.26%) IVM groups and their 24hHOLD counterparts (70.69 ± 2.37% and 85.56 ± 13.47% respectively). Finally, oocytes directly matured 50h in IVM and their respective 24hHOLD+ 50h-IVM group indicated significantly higher (P≤0.01) PGA blastocyst development rates (16.37 ± 4.07% and 17.04% ± 2.25%) in comparison with the 42h (3.77 ± 2.78%) and 24hHOLD+ 42h-IVM counterpart (2.22% ± 3.85%). Both 50h-IVM groups showed a tendency toward improved PGA blastocyst development compared to the 46h-IVM (9.78 ± 5.24%) and 24hHOLD + 46h-IVM (10.83% ± 1.44%) groups, though the difference was not significant. These findings show that 42h-IVM reduces developmental capacity in PGA pig embryos. Additionally, holding oocytes in H-SOF for 24h before 42h, 46h, or 50h of IVM does not affect developmental rates compared to non-held groups.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CLONING, TRANSGENESIS, STEM CELLS

Optimizing time of lipofection for improved CRISPR/Cas9-mediated genome editing in porcine embryos

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Lipofection has been widely used to introduce external molecules into cells since its development in 1987 by Felgner *et al* (Proc Natl Acad Sci USA, 84, 7413, 1987). We have generated gene edited embryos by this method using the CRISPR/Cas9 system without removing the *zona pellucida* of the oocyte (Piñeiro-Silva, Animals, 13, 342, 2023), but the optimal time of the lipofection treatment has not been studied. Therefore, our objective was to compare three different times of lipofection to generate Calpain3 mutant embryos.

In vitro matured oocytes were lipofected with Lipofectamine CRISPRMAX Cas9 with sgRNA and protein Cas9 for 4h (L4h), 8h (L8h) and 24h (L24h), fertilized and cultured *in vitro* for up to 6 days. The IVF and the lipofection treatment were performed simultaneously. An untreated group was used as control (C) with an IVF time of 24h. Penetration and monospermy rates were evaluated at 24h post-insemination (pi) by Hoechst staining and evaluation under fluorescence microscope. Cleavage and blastocyst rates were evaluated at day 2 and 6 pi. Mutation and mosaicism rates were analyzed by fluorescent PCR-capillary gel electrophoresis. Overall efficiency was also evaluated (mutant embryos/total oocytes). 619 oocytes were analyzed for IVF (C:149, L4h:153, L8h:155, L24h:162) and 700 for embryo development and gene mutation (C:169, L4h:176, L8h:175, L24h:180). 3 replicates were performed. Data were analyzed by a Kruskal–Wallis test and compared by a Conover–Inman test.

The penetration rate was higher in C group in comparison with L4h group (95.5±1.8% vs. 82.4±3.3%, $p<0.05$), with intermediate values in the other groups (L8h:88.2±2.8%, L24h:89.2±2.6%). Monospermy was similar in all groups (C:52.3±4.4%, L4h:47.3±4.7%, L8h:41.2±4.5%, L24h:49.2±4.4%, $p=0.35$). The cleavage rate was lower in the L4h and L24h groups compared to C group (42.6±3.7%, 49.4±3.7% vs. 61.5±3.8%, $p<0.05$), but blastocyst rate was similar in all groups (C: 27.8±3.5%, L4h: 21.0±3.1%, L8h: 29.7±3.5%, L24h: 21.7±3.1%, $p=0.15$). The mutation rate was lower in the L24h group compared to the L8h group (27.0±7.4% vs. 49.0±7.1%, $p<0.05$), with intermediate values for L4h group (43.2±8.3%). The overall efficiency followed the same pattern, with higher values for L8h in comparison with L24h (14.4±2.7% vs. 5.6±1.7%, $p<0.05$) and intermediate values for L4h (9.1±2.2%). The mosaicism rate was similar for all groups (L4h: 50.0±12.9% n=8/16, L8h: 40.0±10% n=10/25, L24h: 20.0±1.3% n=2/10, $p=0.32$).

Considering these results, the CRISPR/Cas9 system is able to enter the oocyte in the first 4 hours of coincubation with lipofectamine but reaches the highest efficiency at 8 hours of coincubation. L4h had lower penetration and cleavage rates due to the shorter sperm-oocyte coincubation time. In addition, lipofectamine may have a toxic effect, as the cleavage and mutation rates decreased with longer lipofection time. For these reasons, lipofection can be an effective method to produce genetically modified animals and embryos, but optimization of the process is still needed to explore the potential application of this method.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CLONING, TRANSGENESIS, STEM CELLS

Successful generation of mitotically stable lines of chicken blastoderm-derived embryonic stem cells

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Pluripotent embryonic stem cell (ESC) lines established from the blastoderm, which had been isolated from embryonic discs of the domestic fowl (*Gallus gallus domesticus*) embryos at the stage X not only serve as an innovative model for studying avian embryonic development but are also gaining increasing importance, both in the context of safeguarding various populations outside their natural environment and in preserving the genetic diversity of poultry species and breeds. The purpose of the current study was to develop an efficient strategy for: (1) isolating chicken blastodermal cells (BCs); (2) generating stable BC-derived ESC lines under *ex ovo* conditions; and (3) identifying their pluripotency-related proteomic profiles based on the expression of such biomarkers as Oct-3/4 (octamer-binding transcription factor-3/4, also designated as POU5F1; a member of the family of POU (Pit-Oct-Unc)-domain- and homeodomain-containing transcription factors) and Sox2 (sex-determining region Y (SRY)-box 2; a member of the high mobility group (HMG)-box family of DNA-binding transcription factors). ESCs, which had been established from BCs isolated from a total of 45 fertilized eggs at the stage X (n = 3), were cultured for a minimum of 15 passages under *ex vivo* conditions. During the *ex-ovo* expansion of blastoderm-derived ESCs, their adhesive and proliferative capabilities were compared depending on the type of vessels used for *in vitro* culture (8-well Ibidi glass-bottomed microplates coated with synthetic polymeric substrate/poly-*L*-lysine and cell culture dishes comprised of polystyrene plastic and coated with type I collagen - SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes). By using Western-blot analysis and immunofluorescence staining, the protein expression profiles have been investigated for the pluripotency-related biomarkers that have been represented by the members of a family of homeobox (Hox) transcription factors (Oct-3/4 and Sox2), which display the presence of homeodomain, i.e., a conserved 60-amino acid helix-turn-helix motif-containing and DNA-binding domain. Throughout the extended *in vitro* culture, chicken B-ESCs have been found to maintain the typical morphology of embryonic stem cells. Moreover, the expression of selected pluripotency-related markers of stemness was confirmed at the protein levels based on the detecting the presence of such homeotic (homeodomain/homeobox-containing) transcription factors as Oct-3/4 and Sox2. Conclusively, the use of 8-well Ibidi glass-bottomed microplates coated with poly-*L*-lysine and SPL Life Sciences SPL Coat™ Collagen Type I Coated Dishes exerted a comparable advantageous impact on the proliferative capabilities of chicken BC-derived ESC lines.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

The potency of Moringa oleifera leaf extract inclusion in tris egg yolk extender of bull epididymal semen during cryopreservation

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The post-slaughtered bull semen has been described to be extremely susceptible to cold shock and sensitive to peroxidative damage as a result of the high content of polyunsaturated fatty acids found on the sperm membrane. Owing to this, the reactive oxygen species (ROS) and free radicals stress affects cryopreservation outcomes. During cryopreservation, semen extender is required to enhance the improvement of semen qualities. Semen extender can be supplemented with Moringa oleifera leaf extract (MOLE) as the source of natural antioxidant which is required to reverse the development of ROS caused by imbalances between the ROS activities and amounts of antioxidants in the semen. The extract from MOLE is used because of its accessibility to many farmers is South Africa. The study evaluated the effect of different MOLE concentrations levels of 0; 4; 8 and 12% supplemented in the tris-egg yolk semen extender during cryopreservation of epididymal bull semen. Bull testicles (n=50) were randomly collected from the local slaughterhouse and transported (5°C) to the laboratory. The recovery of epididymis and flushing of sperm was done within an hour of collection. Furthermore, semen was diluted with tris-egg yolk extender (TEY- fraction A) and equilibrated for 120 min, and later supplemented with TEYE- fraction B containing different level of MOLE: TEYE+MOLE 0% (control), TEYE+MOLE 4%, TEYE+MOLE 8% and TEYE+MOLE 12%) and then loaded into 0.25 mL straws before freezing. Thawing of frozen semen straws was done for 10 sec in air and 1 minute inside the warm water (37°C), and the semen samples were evaluated for sperm viability and abnormalities% per treatment groups using a computer-aided sperm analyser system. Data was analysed using the General Linear Model procedures of the Minitab statistical package of 2019. There was a significant effect of TEYE+MOLE levels (0; 4, 8 and 12%) on epididymal sperm rapid motility (50.31±1.4; 54.06±2.3; 57.94±2.7; and 53.63±1.8; respectively) and sperm viability% (45.25±3.4; 53.63±4.0; 58.63±4.4 and 56.44±2.5, respectively; $P<0.05$). The TEYE+MOLE8% had a better sperm motility and viability% as compared to other treatments groups. No significant difference was recorded on sperm morphology% amongst all levels of TEYE+MOLE. In conclusion, supplementing of MOLE extracts in the tris-egg yolk semen extender was able to maintain better epididymal sperm motility and viability recovery following thawing.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Relationship between oocyte mitochondrial activity and female age in mares

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In animal reproduction, dams are not usually required to produce offspring at advanced age. However, for mares of high genetic value, owners are increasingly asking for obtaining embryos from older animals. A decrease in mitochondrial activity, in oocytes recovered from advanced maternal age women, led to spindle assembly alterations, reduced levels of proteins responsible for chromosomes pairing and segregation, and telomere shortening. This study aimed to understand the relationship between female age and mitochondrial activity in the horse. Oocytes were recovered by follicular fluid aspiration from ovaries of young (<15 years; N° mares = 43) and aged (>15 years; N° mares = 46) slaughtered mares. All ovaries have been recovered only from healthy mares. Media, supplements, antibodies were purchased from Sigma Aldrich (Milan, Italy) unless otherwise stated. Oocyte were matured in DMEM-F12+10%FBS (Gibco, ThermoFisher Scientific, Waltham, USA) +50 ng/ml EGF+100 ng/ml IGF-1+0.1 IU/mL FSH-LH (Pluset, Calier, Como, Italy) at 38.5°C and 5% CO_2 for 30 hrs. After IVM, oocytes were fixed in 4% paraformaldehyde, then the zona pellucida was removed for immunofluorescence (IF). Blocking was done with goat serum (GS) in Tween 20+BSA (PBS-TB) followed by an overnight incubation at 4°C in PBS-TB+GS+DLAT antibody (to detect mitochondrial activity) +TOMM20 antibody (to identify mitochondrial distribution), and a second incubation with anti-mouse AF488 and anti-rabbit AF568 antibodies in PBS-TB. Oocytes were examined by confocal microscopy (Dragonfly High Speed Confocal Microscope System equipped with Fusion program) and images were processed by FIJI ImageJ. CTCF (corrected total cell fluorescence) formula (CTCF (pixel) = Integrated Density – [Area of selected cell X Mean fluorescence of background readings]) was used to calculate cell fluorescence. Data, expressed as mean± standard deviation, were analyzed for normal distribution, using a Shapiro Wilk test, and processed by a two tailed T-test using Wizard 2 (Version 2.0.16). Significance was assessed for $p<0.05$. A total of 78 matured oocytes were used, 34 from young mares and 44 from aged ones. Some of them served to adapt a human IF protocol to the horse, thus 23 (young) and 38 (aged) oocytes were finally analyzed. The average fluorescence registered for DLAT, was significantly higher in oocytes recovered from young (254086.8 ±385910.8) than aged mares (103494.1±34065.3; $p<0.05$). However, despite the apparently higher number of mitochondria revealed by TOMM20 in oocytes from older females, the values were not statistically different (young 15944.7±17785.7 vs aged 34065.3±57579; $p>0.05$). These findings open exciting prospects for future studies and clinical applications. Deepening the molecular mechanisms underlying the decrease in mitochondrial activity in oocytes from aged mares might be useful to identify new strategies to improve oocyte quality and increase the chances of success in assisted fertilization techniques.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Development of bovine vitrified oocytes can be improved by antioxidants added during post-warm recovery period

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Vitrification of bovine oocytes can impair further embryo development mostly due to oxidative stress. Various exogenous antioxidants (ascorbic acid, N-acetyl cysteine, melatonin, resveratrol, niacin, coenzyme Q10 and others), added either during the maturation, or closely prior to vitrification, have been shown to ameliorate the post-warm oxidative stress in mouse, sheep and bovine oocytes with a variable success. The aim of this study was to verify whether a relatively short recovery culture of oocytes post-warming in presence of chosen antioxidants (glutathione and astaxanthin) is enough to improve their post-warm survival and development. Bovine *in vitro* matured oocytes (n= 905) were washed in the vitrification medium (M199, 10% foetal bovine serum (FBS), 30% ethylene glycol, 1 M sucrose), placed onto electron microscopy grade grids and vitrified using ultra-rapid cooling technique. Following warming, the oocytes were incubated for 3 hours (post-warm recovery) in the maturation medium (M199, 10% FBS, 0.25 mM sodium pyruvate, 50 µg/mL gentamicin, 1 I.U FSH/LH (Pluset) supplemented with either glutathione (GSH; 5 mM; n=223) or astaxanthin (AX; 2.5 µM; n=226) or none (Control; n=456). Vitrification significantly induced the formation of ROS (CellROX fluorescent staining) in oocytes, while GSH reduced this value ($p<0.05$) in post-warmed oocytes. GSH, although did not increase the total blastocyst rate (Day 6-Day 8: 20.20% vs. 17.49% in control), but it increased the proportion of faster developing blastocysts (Day 6: 23.07% vs. 13.5%; Day 7: 48.7% vs. 42.5%, resp.), reduced the apoptosis incidence (TUNEL assay) up to the control level and reversed harmful impact of vitrification on actin cytoskeleton structure (phalloidine-TRITC staining). AX reduced ROS formation and lipid peroxidation (BODIPY staining) in vitrified oocytes. Development to the blastocyst stage in vitrified oocytes (D6-D8 blastocysts: 15.34%) was not improved by AX (17.3%). Nevertheless, AX promoted blastocyst proliferation (DAPI staining; total cell number - 105.28± 4.45) compared to vitrified group (94.03±5.08) and showed trend of improving the actin cytoskeleton quality. RT-qPCR assay revealed that AX stimulated expression of development-related genes (*GJB5, CAT* and *GPX4*) and suppressed pro-apoptotic *CAS9* gene expression. In conclusion, glutathione confirmed its protective action against vitrification-induced damages. Astaxanthin during post-warm recovery period reduced oxidative stress (ROS) in vitrified oocytes and improved quality of blastocysts. These results suggest that even a short recovery culture of bovine oocytes post-warming in presence of antioxidant(s) can improve their development.

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Conservation of bovine ovarian cortex. Impact of cryopreservation procedure and sample size on the morphology of preantral follicles.

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The cryopreservation (CP) of ovarian cortical tissue (OcT) offers an alternative to overcome the lack of an effective oocyte CP method. Isolation and subsequent *in vitro* culture of preantral follicles (PaF) from CP OcT could allow to obtain viable oocytes for *in vitro* embryo production or conservation programs. Slow freezing (SF) of OcT is a more established protocol. Over the last decade vitrification has also developed for OcT, however the need of an effective standardized protocol remains unsolved.

In this work we analysed the effects of SF and vitrification on the PaF morphology. Thus, bovine OcT pieces of 10mm x 10mm x 1mm (large) or 10mm x 5mm x 1mm (small) from 3 animals were frozen-thawed (FT) or vitrified-warmed following two protocols (VW1 & VW2).

SF up to -70ºC was performed in PBS+4g/L BSA+12% DMSO following a three-step protocol at room temperature (RT). Then cryovials were stored in liquid nitrogen (LN). For thawing, the cryovials were thawed in a water bath at 42ºC, placed in ice and cryoprotectant was removed by serial dilution with PBS+4g/L BSA.

OcT samples (VW1 & VW2) were vitrified in 1.8 ml open cryovials. VW1 samples were incubated at RT in PBS + 20% FCS (HM) + 5% ethylene glycol (EG) + 5% DMSO + 0,125M sucrose (V1S1; 5 min) and then in HM + 10% EG + 10% DMSO + 0,25M sucrose (V1S2; 2 min). Finally, samples were moved to HM + 20% EG + 20% DMSO + 0.5 M sucrose (V1S3; 3 min) and vitrified in LN. For warming, samples were incubated at RT in V1S2 (5 min) and in V1S1 (5 min), and washed in HM (5 min).

VW2 samples were incubated in in HM + 7.5% EG + 7.5% DMSO + 0.5 M sucrose at RT (25 min) and vitrified in HM + 20% EG + 20% DMSO + 1 M sucrose (15 min). For warming, samples were incubated in HM + 1M sucrose (1 min; 37ºC), in HM + 0.5M sucrose (3 min; RT) and in HM (5 min; RT).

Fresh control (Ctrl), FT, VW1 and VW2 OcT pieces were fixed, dehydrated, embedded in paraffin, and serially sectioned (5 µm). Follicle morphology and distribution was examined in H/E slides. PaF were classified as collapsed (showing ooplasm shrinkage or detachment of the basal lamina) or morphologically normal (MNF) (Herraiz *et al.*, Fertility and Sterility 113:609, 2020). A total of 1,454 PaF were counted (Ctrl: 377; FT: 340; VW1: 400; VW2: 337). Data were analysed by a Pearson's Chi square.

VW2 significantly improved the percentage of MNF compared to VW1 (66.37 *vs* 29.83 and 65.79 *vs* 26.94, for large and small samples respectively; $p<0.001$) with any used sample size.

When the percentage of MNF in large FT samples was compared with Ctrl, it was significantly reduced (51.27 *vs* 85.71, respectively; $p<0.001$). Interestingly, FT OcT and Ctrl small samples showed similar percentages of MNF (88.11 *vs* 78.89, respectively; $p>0.05$).

The total percentages of MNF were similar among groups and when specific follicular subpopulations (primordial, primary and secondary) were examined.

Although CP negatively affected follicular integrity, further studies are needed for evaluating the functionality of the surviving PaFs.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Effects of cold storage and different cryopreservation methods on canine epididymal sperm

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Recent years have seen a drastic decline in biodiversity. In addition to the traditional in vitro conservation of genetic material, there is a need to establish a well-functioning protocol to cryopreserve the genetic material of a male of an endangered species or with valuable genetics. Storage of epididymal sperm is a feasible method to achieve this goal, however, time of sample delivery and freezing technique are key factors affecting the success rate. Our aim was to investigate the freezing ability of canine epididymal spermatozoa in fresh stage and after 24 hours storage at 4°C with two different freezing protocols (ultra-rapid freezing [UR] and vitrification [VF]). Testes were collected from 15 castrated mixed-breed dogs. Sperms were obtained from cauda epididymis immediately after collection or after 24h storage, using the incision method. Collected sperms were cryopreserved with UR and VF. The following parameters were evaluated: total and progressive motility, normal morphology rate, rate of acrosome defects, rate of detached heads, rate of tail defects and proAKAP4 concentration. Concerning the total motility, no significant difference was found between the UR and fresh groups. There was no significant difference in the progressive motility between the UR 24h group (32.8% ± 22.8) compared to the fresh groups (Fresh 0h [49.7% ± 17.2] and Fresh 24h [56.3% ± 22.7]), but significantly lower progressive motility was found in the UR 0h group [14% ± 10.4] compared to both Fresh 0h and 24h. However, significantly lower total and progressive motility in VF groups (<10%) were found compared to both fresh and UR groups. Regarding the morphology analysis, UR and VF resulted in significantly lower rate of normal morphology than that of Fresh (19.4 ± 7.5, 29.4 ± 9.8 and 44 ± 11.8, respectively). A significant effect of the freezing method was also observed in acrosome defect rate (13% ± 7.8; 26% ± 12.8 and 29.7 ± 16.4 in Fresh, UR and VF, respectively). In case of detached head and tail defects, in addition to incubation, the freezing method had no effect. Assessing the proAKAP4 level, higher overall concentration was found in fresh and VF groups than that of UR group, which resulted in higher proportion of excellent (100%, 17% and 63.6% in Fresh, UR and VF, respectively) quality samples. We found that total motility after 24 hours storage and ultrarapid freezing was not statistically different from fresh samples. Despite the low motility,vitrification can preserve the high proAKAP4 concentration, which is a key factor in semen quality. However, more studies are needed to clarify this finding. These data indicate that one day storage and cryopreservation of epididymal sperm of a suddenly dead male can be a feasible method for fertility preservation, providing suitable samples for genetic conservation.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

CRYOPRESERVATION AND CRYOBIOLOGY

Canine preantral follicle cryopreservation: comparison of slow freezing, open and closed vitrification.

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There is an increased interest and practical potential in the application of ovarian preantral follicles (PAF). Follicle in vitro culture (FIVC) could provide access to high numbers of oocytes which can be matured and fertilized in vitro. Moreover, cryopreservation (CP) of PAFs can be efficient in mammalian gene preservation, regardless of the age or reproductive phase. The aim of our study was to find the best method to cryopreserve canine preantral follicles.

Ovaries were collected from ovariohysterectomized bitches (N= 20; undefined crossbreds) being in different ages. Ovariohysterectomies were carried out on multiple days (10 independent culture trials). After collection, ovaries were placed in sterile 50 ml centrifuge tubes, containing PBS + 10% bovine serum. Samples were stored at room temperature and delivered to the laboratory within 2 hours. The ovarian cortex of each ovary was sliced to approximately 1 mm^2 pieces with a surgical blade, then placed in digestive solution (HEPES-modified Medium 199 + 3 mg/ml collagenase) and incubated for 90 minutes at 37 °C. Following the enzymatic digestion, preantral follicles were isolated manually with 28G needles attached to 1 ml syringes. After isolation, morphologically normal secondary follicles were selected and randomly divided into four groups: fresh control (FR), slow freezing (SF), open vitrification (with open pulled straw; OPS) and closed vitrification (with cryotube; CT).

Live cell rate of follicles was analyzed immediately after isolation (FR) or thawing (SF, OPS and CT). Then, PAFs of each group were cultured in vitro for 10 days in 20 µl drops of FSH-supplemented medium (OptiMEM) at 38.5 °C and 6.5% CO2. Survival rate, area change, and estradiol production were examined. Live cell rate (determined by calculating calcein-AM positive cells) of follicles was similar to FR (83.6%±17.6) in OPS (80.3%±23.5) and SF (93%±3) groups, instead of CT, where significantly lower rate was found (58.7%±28). PAF survival rate during the IVC was lower in all of the cryopreserved groups than in fresh (82.2%, 85.2% and 37.5% in CT, OPS and SF, respectively; vs. 98.4% in FR). Fresh follicles showed continuously increasing area and estradiol production from Day 2 to 10 of IVC, while PAFs of OPS and SF increased their size until Day5. CT stopped their growth after Day2. Estradiol production was elevated continuously in FR and OPS throughout the IVC period (41.6 to 105 pg/ml, and 37.4 to 42.4 pg/ml, respectively; from Day2-10, median values), while remained unchanged in CT and SF follicles (28.2 to 27.9 pg/ml, and 27.49 to 31.9 pg/ml, respectively).

Our data show that open vitrification is superior to other cryopreservation methods to preserve canine isolated PAFs, however, refinement of the system is needed.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL 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 meiotic spindle and ROS levels

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Ethylene glycol (EG) and dimethyl sulfoxide (DMSO) have been established as cryoprotectants (CPAs) for vitrification/warming of bovine *in vitro* matured (IVM) oocytes (García-Martínez *et al*, Theriogenology 184, 110-23, 2022). Given DMSO's cytotoxicity, propylene-glycol (PG) has been proposed as an alternative CPA. This study examined the effects of vitrifying IVM bovine oocytes with PG or DMSO combined with EG on spindle morphology and reactive oxygen species (ROS) production after warming. Oocytes were IVM for 21h and vitrified using *in silico* designed protocols: 7.5%DMSO-7.5%EG for 2min30sec and 15%DMSO-15%EG+0,5M sucrose for <1min (VIT-DMSO) or 7.5%PG-7.5%EG for 1min35sec and 15%PG-15%EG+0.5M sucrose <1min (VIT-PG). Oocytes were warmed and allowed to recover for 3 additional hours. Fresh, non-vitrified IVMoocytes served as control and IVM oocytes exposed to vitrification/warming solutions but non-vitrified/warmed served as controls for CPA cytotoxicity (CPA-DMSO or CPA-PG). Spindle morphology was assessed at 24h of IVM. Intracellular ROS levels were quantified by labeling with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and assessed after warming (0h) and at 24 h of IVM (3h). Relative ROS levels was used as a variable measure to classify intensity levels. All procedures were performed as described in García-Martínez *et al*. (Int J Mol Sci, 21, 7547, 2020). Data were statistically analyzed with GraphPad Prism; Shapiro–Wilk was used for testing normality, Levene's test for homogeneity of variance followed by one-way ANOVA or a Kruskal-Wallis test (p<0,05). Oocytes vitrified with EG+PG showed similar percentages of oocytes reaching the MII stage (VIT-PG:78.6%±1.7 n=70) than the control fresh group (Control: 81.3%±1 n=123) while the other groups showed significantly lower percentages .(CPA-PG:62.7%±2.3 n=59; CPA-DMSO: 71.9%±1.9 n=57; VIT-DMSO: 77.4%±1.2 n=62). Although lower (P<0.05) than the Control fresh group (80%±0.7 n=123), no differences in percentages of normal spindle configuration were observed among vitrified groups (VIT-DMSO: 75%±1.8 n=62; VIT-PG:74.6%±2.1 n=70). Exposure to EG+DMSO resulted in lower (p<0.05) percentages of normal spindle configuration (CPA-DMSO: 73.2% ±2.3% n=57) compared to the control group, but significantly higher percentages were observed after exposure to EG+PG (CPA-PG: 89.2%±0.8 n=59). At 0h post-warming, vitrification with EG+DMSO resulted in higher relative ROS levels (12±0.9 n=56) than at 3h post-warming (6.1±0.1 n=52). No differences in relative ROS levels were observed in the VIT-PG group (0h: 6.5±0.2 n=42; 3h: 6.8±0.2 n=43) or CPAs groups (CPA-DMSO 0h:7.2±0.3 n=39; 3h:6.4±0.1 n=44; CPA-PG 0h:6.6±0.2 n=27; 3h:5.9±0.1 n=35). Results showed that vitrifying IVM bovine oocytes with EG+PG had no effect on spindle morphology when compared to the use of EG+DMSO, but it did reduce ROS levels after warming. These findings highlight PG's potential as a CPA, but further research on embryo development after vitrification of IVM bovine oocytes with EG+PG is required to confirm the efficiency of this CPA.

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THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPPORT BIOTECHNOLOGIES: DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY AND OMICS-TECHNOLOGIES

Utilizing ultrasonographic echotexture as a diagnostic tool to assess postpartum uterine endometrial involution

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The present study aims to utilize computer-assisted ultrasonographic echotexture analysis (BS200 Pro® Image processing and analyses software, BAB, Ankara, Turkey) as a diagnostic method to evaluate the histological involution of the uterus in postpartum cows. The study included a total of 27 cows from three different breeds (Holstein, Brown Swiss, and Simmental) and varying parities (1st, 2nd, and 3rd). The breed and parity factors were added to the statistical design. The study timeline was divided into eight distinct periods ranging from day -15 (pre-partum) to day 42 (postpartum). The researchers employed ultrasound measurements to assess the cervical (CD) and uterine horn diameters (UHD), and they collected ultrasonographic images of the uterine endometrium on different days for echotexture analysis. These images were analyzed using a computer-assisted echotexture program, which measured various parameters including mean gray level (MGL), gradient (GR), homogeneity (HOM), and contrast (CON) values. Throughout the study, clinical assessments and pH measurements of vaginal discharges were conducted on the same day intervals. Additionally, on days 21, 28, 35, and 42, samples from the endometrium were taken for cytological examination. Blood samples were collected from the cows on all study days to determine serum calcium, phosphorus, and magnesium levels. Blood beta-hydroxybutyric acid (β-HBA) levels were measured on days 7 and 21. The study found that MGL, GR, HOM, CON, CD, and UHD of the cows exhibited significant variations on different study days. In healthy cows, MGL and GR levels decreased from day 1 to day 21, and then increased until day 42. Problematic cows showed differences in MGL, GR, HOM, and CON values on various examination days. Comparing the overall average of all days, healthy cows had lower HOM, CD, and UHD, while they had higher MGL, GR, and CON values when compared to problematic cows. The study established ROC cut-off values of GR (12.01), HOM (0.055), and CON (37.19) on day 35 of postpartum for this purpose. In conclusion, the research indicates that computer-assisted ultrasonographic echotexture analysis may serve as a valuable tool for evaluating endometrial uterine involution in postpartum cows. This could help distinguish between a healthy and problematic uterine endometrium, ultimately impacting the success rates of first insemination in postpartum cows.

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SUPPORT BIOTECHNOLOGIES: DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY AND OMICS-TECHNOLOGIES

Effect of heat stress on proteome and transcriptome of in vitro cultured bovine oviductal epithelial cells.

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Heat stress is known to reduce fertility of cattle. Increased body temperature affects oocyte and embryo development directly or indirectly through alterations of the maternal reproductive tract. Heat stress-induced modifications of the oviductal microenvironment may disturb embryonic development and cause embryonic death. This study aimed to identify short-term heat stress-induced changes in bovine oviductal epithelial cells (BOECs) and their secretory profiles under *in vitro* conditions. Oviducts ipsilateral to the corpus luteum at Day 1 to 3 of the oestrous cycle were collected at a slaughterhouse and transported to the laboratory on ice. The oviducts were gently squeezed using tweezers and recovered BOECs of each oviduct were divided into three groups at a concentration of 10^6 cells/ml and cultured for 24 hours in DMEM/F-12 medium (Gibco 21041-025) supplemented with 0.1% FCS. The first group was incubated for 12 hours at 40.5°C followed by 12 hours at 38.5°C (HS12 group), the second group was incubated for 24 hours at 40.5°C (HS24 group) and the third group was incubated at 38.5°C for 24 hours (control group). After 24 hours, BOECs ($2x10^6$) and conditioned media (2 ml) were collected separately and frozen. In total, 9 samples of BOECs (3 of each group) were submitted for RNA sequencing to detect changes in their gene expression profile. In each group (n=3), the protein of the conditioned media (n=7) was purified, and 10 µg were analysed using nanoLC-MS/MS to identify secreted proteins. Approximately 20,000 genes were analysed with RNA-Seq and the expression profiles were compared between groups. Heat-treated groups HS12 and HS24 showed differences in the mRNA expression of eleven genes compared to the control group, each after Bonferroni correction. Subsequently, genes which were highly significantly differentially expressed in RNA-Seq were selected for validation by qPCR. Various heat shock proteins, e.g., HSPA6, HSPA1A, and HSPH1, were found up-regulated in the HS24 group. Using mass spectrometry, 600 to 900 proteins could be identified in the conditioned media. The protein composition was analysed using ANOVA. Overall, only a few variations were found in the HS24 group and even less in the HS12 group. These proteins with tendentially different expressions can be assigned to the functional groups chaperone binding, stress response, growth factor binding and protein folding. In conclusion, BOECs cultured *in vitro* showed signs of heat stress in the transcriptome with clearly upregulated heat stress genes in the HS24 group, but less clear results in the HS12 group. In the protein secretome profile, few effects of heat treatment were evident. Further analysis of data is planned to identify proteins with potential influence on early embryo development.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPPORT BIOTECHNOLOGIES: DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY AND OMICS-TECHNOLOGIES

Integrative analysis of microRNA and mRNA transcriptomes revealed dysregulation of TGF-beta signaling pathway in SOPS-vitrified porcine blastocysts

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Vitrification is the only effective method to cryopreserve pig embryos. The superfine open pulled straw (SOPS) method provides excellent in vitro viability post warming with blastocysts derived in vivo. However, higher pregnancy loss has been observed after transfer of vitrified embryos when compared to transfers with fresh embryos. The aim of this study was to investigate how SOPS vitrification of pig blastocysts affects the expression profile of microRNA (miRNA) transcriptome, as well as its relation to changes in the expression of target genes (TGs) in the vitrified blastocysts. In vivo derived porcine blastocysts were collected surgically and vitrified using the SOPS method (n = 60) as described before (Cuello et al., Sci Rep 6:33915, 2016). Embryos were cultured in vitro for 24 h after warming. Fresh blastocysts (n = 60) cultured for 24 hours served as controls. After in vitro culture, five pools of eight viable blastocysts from each group were prepared for analysis based on a microarray approach for miRNA (GeneChip miRNA 4.1 Thermo Fisher Scientific) and mRNA (GeneChip® Porcine Genome Array, Affymetrix) expression. Then, an integrative analysis of miRNA and mRNA transcriptomes data were performed with the Transcriptome Analysis Console 4.0.2 software. Biological interpretation of data was investigated using the Partek Genomics Suite and Pathways software. A threshold of 1.5-fold change and $p<0.05$ was used to identify differentially expressed (DE) miRNAs and DE TGs. Survival after 24 h of in vitro culture was similar for vitrified blastocysts (96.7%) and the controls (100%). The vitrified blastocysts had 94 (one upregulated and 93 downregulated) DE miRNAs compared with the controls, one of them (miR-503) was annotated for Sus scrofa. The altered miRNAs identified in this study were related mainly to cell proliferation, apoptosis, and the response to cell stress. Microarray analysis showed 210 (44 downregulated and 166 upregulated) DE genes in vitrified blastocysts compared to the control group. A total of 27 DE genes were found to be TGs regulated by the DE miRNAs identified in this study. Gene Ontology term analysis revealed that the DE TGs were associated mainly with biological processes such as reproductive process, cell population proliferation and growth. Some of these TGs were significantly involved in the TGF-beta signaling pathway, which is essential for embryo development, implantation and placentation. Dysregulation of miR-548a-3p and miR-4685-5p was also observed in the vitrified blastocysts. These miRNAs were associated with the overexpression of the ZFP36L1. This DE TG also plays a key role in embryonic development. In summary, vitrification via the SOPS system dysregulates miRNAs. Further studies are needed to clarify the consequences of dysregulation of miRNAs and TGs involved in the TGF-beta and the potential impact of this dysregulation in implantation and pregnancy. Supported by MCIN/AEI/10.13039/501100011033 and ERDF (RTI2018-093525-B-I00), Spain; Seneca Fundation (19892/GERM/15), Spain.

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THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

SUPPORT BIOTECHNOLOGIES: DIAGNOSIS THROUGH IMAGING, MOLECULAR BIOLOGY AND OMICS-TECHNOLOGIES

Imaging mitochondrial Hydrogen Peroxide in bovine oocytes and early embryos using a novel ratiometric sensor: a preliminary study

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Reactive oxygen species (ROS) are a wide range of molecules known to trigger oxidative stress (OS). OS is one of the main factors impairing embryo quality when oocyte maturation, fertilization, and early embryogenesis are performed in vitro. Nevertheless, the role of ROS as key regulators of physiological mechanisms is also widely accepted, leading to the general assumption that excessive ROS suppression could alter cellular functions (Tsunoda, Reprod Med Biol 2014;13:71). A better understanding of the role of each ROS is needed to modulate culture systems to improve reproductive efficiency. An issue in this type of studies is the limited ability to track and quantify fluctuations of each ROS with high resolution in space and time (Sies, Nat Rev Mol Cell Biol, 2022; 23:499). To start to address this issue, we tested the suitability of a novel ultrasensitive fluorescent ratiometric sensor (Hyper7), capable of monitoring H_2O_2 fluctuations in vivo (Pak, Cell Metabolism 2020; 31:642), to detect changes in H_2O_2 levels in bovine oocytes and zygotes, as previously done in the Xenopus (Han, Cell Rep 2018; 22:21). In our experiments, mRNAs encoding the mithocondria (mt)-targeted Hyper7 was microinjected into immature oocytes or in vitro matured ones. The microinjected oocytes were then in vitro matured or fertilized. Preliminary trials revealed that micromanipulation per se did not severely impair the ability of the oocyte to mature, or to be fertilized and reach the blastocyst stage**.** To validate the sensor, a total of 172 oocytes and 154 zygotes were included in the study. Samples were imaged under control conditions or in the presence of a prooxidant challenge (tert-Butyl hydroperoxide). Imaging was conducted at the NOLIMITS microscopy facility of the University of Milan, using spinning disk microscopy equipped with a temperature-controlled CO_2 chamber and appropriate lasers and filters every 30 seconds. Image analysis was conducted after background subtraction to calculate the ratio value of the oxidized versus the reduced form of the sensor as previously described (Han, Cell Rep 2018; 22:21). Data analysis showed that the sensor can detect H_2O_2 in oocytes and zygotes. However, under control conditions, a significant increase was observed after 20 minutes (Friedman test), suggesting phototoxicity as a causal factor during imaging. Nevertheless, both oocytes and zygotes treated with prooxidants exhibited higher values than those in the control group, confirming the sensors' ability to quantify elevated H_2O_2 levels (Two-way ANOVA). On the other hand, additional studies conducted on 75 zygotes revealed that high concentration of a mix of antioxidants (acetyl-L-carnitine, N-acetyl-L-cysteine, and α-lipoic) did not reduce mt-H2O2 but significantly increased its production, suggesting some paradoxical effects. Our study marks the first application of Hyper7 in mammalian oocytes and zygotes, while posing the critical need to manage phototoxicity in subsequent research. Funded by H2020 MSCA-ITN-ETN n.860960 (EUROVA); SEED2019 UNIMI N.1250 (cROSs-Talk) & Piano di Sostegno alla Ricerca: Linea 2 – Azione A (Molecular and structural responses to stressors in different cells and tissue models).Thematic Section: 40th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PRACTITIONER'S AND CLINICAL REPORTS

Colostrum as an indicator for better reproductive performance in dairy cow

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Effective donor cow selection is essential in implementing assisted reproduction in the herd. There is much debate about how to measure cow immunity because it is closely related to cow productivity, health and reproductive performance. The work aimed to analyze the immunoglobulin G (IgG) level in cow colostrum in relation to the parameters of subsequent lactation. The study involved the analysis of various parameters such as 305 days (305P) and total lactation productivity (TP), milk fat (MF), milk protein (MP), somatic cell count (SCC), service (SP) and inter-calving period (ICP), artificial insemination times per pregnancy (AIPP). The IgG level was measured immediately after calving using a Digital Brix Refractometer (*Spectrum Technologies Inc*.). Recording data from the farm management system was used. The research was conducted on a herd of 600 dairy cows with an average milk yield of approximately 10 thousand per cow per year. It was a free stall-keeping system where cows received a well-calculated total mixed ration twice a day, and water was always available. The data was analyzed using *SPSS 21*, and statistical significance was determined using the Mann-Whitney test with a *p*-value less than 0.05. All cows were divided depending on IgG level in the colostrum: minimal level (23% and less, n=58), optimal (more than 23%). The study's results revealed that the IgG level in milk was 22-30%, and it was not affected by seasonal variations or twins ($p>0.05$). IgG in colostrum was not an essential factor regarding 305P, TP, MF, MP and SCC ($p>0.05$). The SP (110.2±40.39 vs 92.5±31.56 days) and ICP (395.1±41.16 vs 373.7±32.01 days) were longer ($p>0.05$) in cows with minimal (23% and less) IgG, and these cows had more AIPP (2.4±1.17 vs 1.4±0.67, $p<0.05$). A statistically significant positive correlation was detected between IgG in milk and the number of cow lactation ($r=0.42$, $p<0.05$), but a mild negative correlation between IgG level and AIPP ($r=-0.45$, $p<0.05$). In conclusion, the level of IgG in colostrum could help reveal cows with a more stable immune system, higher fertility, shorter service and inter-calving period. These cows might be more suitable for the role of donor cows, but the correlation of colostrum IgG levels with ovarian functionality indicators remains to be investigated.

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Animal Reproduction

THEMATIC SECTION: 40TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

PRACTITIONER'S AND CLINICAL REPORTS

Case report - producing embryos from euthanised or slaughtered cattle

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In vitro production (IVP) of bovine embryos does not necessarily need to be started with cumulus-oocyte-complexes (COCs) of living animals. Especially for research, lots of oocytes are mandatory. Therefore, IVP with COCs of slaughtered cattle has been used for decades. In some cases, cattle with a high individual value to their owners (economic or emotional) have to be euthanized or slaughtered due to illness or declining production. If a "final" reproduction of these animals is planned, ovaries can be gained after slaughter or before euthanasia.

Ten cases of post-mortem IVP are reported here in a German IVP lab for cattle from 2020 to 2024. The animals belonged to the following breeds: Brown-Swiss (2), Galloway (3), Holstein Friesian (3), Simmental (1) and Wagyu (1), aged 2 up to 17 years. The last calving was between 2 and 18 months before IVP. The reason for slaughtering or euthanasia were fertility problems in 6 of 10 cases, for example oophoritis, endometritis or subfertility. Orthopedic diseases like septic arthritis, pelvis fracture and tearing of the adductors happened to the other four animals. The diseases endured between three days and one year. Six animals were slaughtered and four ovariectomies were performed before euthanasia. Transport of the ovaries in saline (0.9% sodium-chloride or phosphate buffered saline with heparin) took 72 minutes in average (5 up to 210 min). In most cases, seven out of ten, the ovaries showed few (at most 2) or no follicles and/or corpora lutea on the surface compared to ovaries of healthy, cyclic animals. After slicing between 13 and 120 COCs could be collected, quality grades 1-2, considered as IVF suitable (5th Edition IETS Manual), and 3-4 were on average equally distributed with 28.4 and 25.0 COCs, respectively. A standard IVP-protocol was performed up to day 7 of IVC (IVF = day 0), and cleavage and developmental rates were recorded. Between 3.8 and 84.4% of the presumptive zygotes cleaved to embryos (average 41.5%) and developmental rates were between 0 and 18.5% (average 5.9%). On average 2.5 transferable embryos were produced per animal, but this success varied because in four out of the ten cases no embryo and in one case 12 embryos were obtained. By grading the embryos, stages of morulae as well as early, blastocysts and expanded blastocysts were detected. In the four non-serving cases reproductive diseases (oophoritis, subfertility), a very long calving interval (18 months) as well as a short one (2 month) might have had an impact on the result, possibly related to a low COCs count and/or being of poor quality (grade 3 and 4). In two cases transport of the ovaries to the lab endured longer than 180 min. Duration of the diseases as well as severity of clinical signs were not clearly related to IVP failure in our observations.

In summary, reproductive diseases were the main reason for slaughtering or euthanasia of the animals presented here and this, as well as too long or short calving interval and/or long transport could be the reason for unsuccessful IVP cycles. On the other hand, in six out of ten cases transferable Embryos were produced as a final chance of reproduction for these animals.