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Sperm storage tubules culture: a new approach for reproductive research in avian species

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Keywords: cell culture, avian, epithelial cells, sperm storage tubules.

Sperm storage tubules (SST) are epithelial structures found in the mucosa of distal half of the oviduct of all avian species studied. These tubules maintain and store sperm up to 70 days and this allows fertilization without insemination. The aim of this work was to set up epithelial SST cells culture for future use as an *in vitro* model for oviduct cells-sperm interaction. Hens (*Gallus gallus domesticus,* Unité de Recherches Avicoles [URA], INRA, Nouzilly.) were euthanized with sodium pentobarbital injection. Oviducts were isolated and removed and the uterovaginal villi was manually dissected under stereomicroscopy. The SST area on the top of isolated mucosal villi was dissected, scalped in small fragments, and enzymatically digested in 1µg/ml Collagenase for 10 min at 41ºC. The digested tissue was flushed for 30 times by pipetting. The enzymatic activity was blocked by washing the tissue twice with culture medium. A second enzymatic digestion was performed by incubating the tissue overnight at 4ºC in 1µg/ml Pronase. The tissue was flushed again and the enzymatic activity was blocked. SST were isolated in 2 / 4% Percoll density gradient centrifugation at 2000g for 30 min at 4ºC. An intermediate phase of Percoll column containing SST was harvested before being maintained in Medium 199 containing 10% BFS and Gentamicin, during 30 min at 41ºC for fibroblast attachment. The medium containing SST was distributed in Lab-Tek Chamber Slide System (Nunc). SST were cultured at 37ºC, 5% CO_2 atmosphere, for 6 days. Immunocytochemistry for epithelial cell type confirmation, was performed with overnight incubation with monoclonal primary antibodies anti-Pan-cytokeratin (1:300, Sigma), Tubulin (1:300, Sigma) and Vimentin (1:500, Sigma) and anti-species secondary antibodies. We observed that, at the end of the enzymatic process, 90% dissected SST was isolated. In phase contrast microscopy we observed integral SST as well as individual cells. After 2 days of culture we observed cell migration from SST borders to form a monolayer. Eighty % cells presented epithelial characteristics as demonstrated with Cytokeratin and Tubulin positivity and Vimentin negativity, in Confocal microscopy. The digestion and isolation processes need to be controlled to differentiate the epithelial surface mucosal cells from SST cells. This method is very effective to isolate the SST specific population of cells that can be used in different reproductive and physiological studies for epithelial cell-sperm interaction.

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Live confocal microscopy time-lapse imaging cholesterol inclusion to plasma membrane of mature bovine oocytes prior to vitrification

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Keywords: bovine oocytes, cholesterol, plasma membrane, confocal microscopy time-lapse.

The present study aimed to evaluate the effect of 2 mg/mL of methyl-β-cyclodextrin (MβCD) as a cholesterol loader to change mature oocyte plasma membrane and increase its tolerance to vitrification procedure. In a first set of experiments, a time-lapse imaging by confocal microscopy was conducted to determine at which time the MβCD loaded the cholesterol in the plasma membrane. Once the timing of cholesterol integration to plasma membrane was defined, we evaluated the effects of a pre-treatment with 2 mg/mL of MβCD for 35 min and 1h prior to vitrification on survival and embryo development rates. Analysis in all groups were performed through an ANOVA, followed by the Sidak's post-hoc test. In all cases, significant level was set at $P < 0.05$. *In vitro* matured oocytes exposed to 2 mg/mL of MβCD showed a clear immunofluorescence in the plasma membrane after a minimum of a 35 min and up to 1h pre-treatment. Higher survival rates were observed when oocytes exposed to 2 mg/mL of MβCD pre-treatment for 35 min (60.6%) were vitrified/warmed compared to 1h pre-treatment (47%). Similar results were observed when blastocyst yield was determined. Blastocyst rate on D7 was higher after 35 min MβCD treatment compared to the 1h MβCD treatment (3.1% vs 0%). However, vitrified oocytes showed lower embryo development rates than fresh non-vitrified oocytes (21.1%). Hence, our results warrant further research to be conclusive.

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Recipient pregnancy rates after transfer of vitrified *in vivo* produced ovine expanded or hatched blastocysts

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Keywords: blastocysts, pregnancy, sheep, vitrification.

The development of embryo transfer technology pushed freezing technology in domestic species. Controlled (traditional) slow freezing and vitrification (ultra-rapid freezing) have been the two major techniques used for embryo cryopreservation (Fahy and Rall, 2007). The ultra-rapid technique, such as vitrification, has reduced time and cost of the procedure since it does not require any special equipment and is, therefore, well adapted to routine field use (Baril, 2001). Sheep and goat embryos are able to survive vitrification procedures and with further research this method may provide an economical alternative to the current freezing methods. Shirazi et al., (2010) indicated that the survival of IVP ovine embryos following vitrification progressively increases as the developmental stage of the embryo proceeds. We studied the effect of vitrification on the developmental stage of the embryo monitored after ET. Donor ewes (10 Romanov breed) were used to produce vitrified embryos kept under semi-extensive husbandry conditions and fed on a maintenance diet (ARC 1990). Superovulation was induced by treatment with ovine follicle stimulating hormone, FSH (Ovagen, Immuno-Chemical Products Ltd) that was administered in 8 equal doses at 12-hourly intervals (total dose equivalent to 9 mg NIADDKoFSH- 17) commencing 60 hours prior to the end of progestagen treatment (12 d). Embryos were recovered by the surgical procedure on day 6 following insemination and were assigned on their developmental stage and quality grade according to standards of the International Embryo Transfer Society (Savoy, IL). Within 2 to 4 h after collection, expanded (n=18) and hatched (n=18) blastocysts were first washed in phosphate-buffered saline (PBS) supplemented with 0.5 mM sodium pyruvate, 3.3 mM glucose and 10% FCS. The embryos were vitrified at room temperature (~23 °C) as follows: 10% EG+10% DMSO for 3 min, followed by 20% EG+20% DMSO+0.5 M Sucrose for 30 s, loaded into OPS and directly plunged into LN2. Before ET the embryos were warmed directly by plunging them into tissue culture medium-199 (TCM-199) + 20% foetal calf serum (FCS) at 37 °C for direct dilution. Following the direct dilution, the embryos were transferred as single into synchronised recipient ewes and allowed to go to term. Pregnancy rates were assessed by ultrasound scanning at 50 days after transfer. We used the *chi-square* test to compare *pregnancy rates*. Late stage embryos produced in vivo to the expanded blastocyst stage before cryopreservation had a significant ($P<0.05$) higher (86%) of pregnancy rate than those recorded (60%) for the hatched stage blastocyst. Our results were higher than the results reported by Garcia-Garcia et al., (2005). They worked with in vivo produced ovine embryos in early stages (2-12 cells) and cultured to the blastocyst stage and frozen thereafter. In that study it was reported that blastocyst stage of embryos had a significantly higher viability than their counterparts frozen at earlier cleavage stages, (66.1% versus 23.1%). The results indicate that in vivo produced embryos up to expanded blastocyst stage can be successfully cryopreserved by vitrification while vitrification success decreases when the stage of development reaches hatched blastocyst.

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Effect of the rewarming temperature on survival rate of IVP bovine embryos vitrified in triacetate cellulose hollow fiber incorporated into a new vitrification device

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Keywords: IVP bovine embryos, rewarming temperature, triacetate cellulose hollow fiber, vitrification.

Hollow fiber vitrification (HFV) was introduced by Matsunari et al. (J. Reprod. Dev. 58: 599–608, 2012) as a method for vitrifying groups of mammal embryos and was shown to be effective for cryopreservation of such cryosensitive objects as in vivo and in vitro produced porcine morulae. Due to the standard and simple vitrification procedure HFV method may be perspective for cryopreservation of bovine oocytes and IVP embryos in combination with embryo transfer methods. The objective of this work was to introduce a vitrification device that will allow effective storage of the triacetate cellulose hollow fibers (HF) that become fragile in liquid nitrogen and to show effectiveness of the HFV method for IVP bovine embryos rewarmed at room temperature (22–24°C). IVP bovine embryos were cultured in modified SOF medium. Morphologically normal blastocysts and expanded blastocysts were collected at day 7 after IVF and used for vitrification. A vitrification device was constructed by connecting a piece of HF to a heat-pulled tip of a glass capillary. A protective sheath was fitted directly on the capillary. Embryos in groups of 5–10 were loaded into the vitrification devices in the equilibration solution containing 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO). Then HFs were exposed to vitrification solution containing 15% EG, 15% DMSO and 0.5M sucrose for 1 minute and immersed into liquid nitrogen. HFs were rewarmed in the solution with 1M sucrose at either 39 °C or 22–24°C and were transferred into dilution and washing solutions stepwise. Rewarmed blastocysts were cultured for 72 hours in modified SOF medium. Reexpansion and hatching rates of embryos were assessed at 24 and 72 hours post rewarming, respectively. Results are presented as mean ± SD. Data was analyzed using Student's t-test. Significance was set at $p < 0.05$. After storage in liquid nitrogen for 2–12 months and transportation to the farm all HFs within vitrification devices remained intact. Volume of vitrification solution with loaded blastocysts within hollow fibers ranged between 0.024 and 0.030 mkl. Reexpansion rates of the blastocysts after rewarming at 22–24°C and 39°C were 82.65 ± 11.62% (159/191; 14 repeats) and 88.73 ± 5.99% (64/73; 7 repeats), respectively. Hatching rates were 53.77 ± 22.37% after rewarming at 22–24 °C and 64.74 ± 12.74 % at 39°C. There were no statistically significant differences between two experimental groups. The introduced vitrification device is relatively simple in construction and protects HFs with loaded embryos from mechanical damage. Due to the very small volume of the samples within HFs, rewarming at room temperature did not significantly affect survival rate of the embryos. Rewarming at 22–24°C can be advantageous for practical uses and may help to avoid temperature related effects of high concentrations of cryoprotectants, such as DMSO.

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Born Simmental calves after the transfer of genetic evaluated day 7 bovine embryos

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Keywords: cattle, day 7 embryos, genetic evaluation, Simmental calves.

Genomic selection can theoretically take place in any stage of an animal's life. The use of ET can increase the intensity of selection, but at the cost of keeping many recipients. This can be avoided by transferring only embryos with desired gender and best breeding values. Improvement of embryo micromanipulation and DNA amplification techniques allows for the direct genetic analysis of bovine embryos prior to implantation. The aim of our study was to set up and optimize a whole embryo production and evaluation line in Simmental cattle to determine gender, polled status, hereditary defects and reliable breeding values on blastomeres at the morula and blastocyst stages. For embryo recovery (n=45) German Simmental animals (n=17) were superovulated using a standard protocol. Embryos were biopsied immediately after recovery by a single operator under a mobile stereo microscope (Olympus) at 50x magnification with a single use special steel blade mounted on a blade holder (Bausch & Lomb, Germany) attached to a micromanipulator (Eppendorf, Germany). Two biopsy methods were compared, first embryos were splitted and one third of a half cut off (G1, n=161) or by cutting of the trophoblast (G2, n=146). Biopsied cells, approximately 10-15, were immediately used for whole genome amplification (*Repli-g* mini Kit, Qiagen) followed by PCR analysis of gender and polledness. Hereditary defects were analyzed using a 5'-exonuclease assay. Embryos were transferred to recipients after *in vitro* culture in SOF supplemented with 5% ECS, 40 µl/ml BME and 10 µl/ml MEM in four-well dishes, under mineral oil, at 39°C and gas mixture (5% CO2, 5% O2, 90% N2) for 24 h. DNA of the first 14 born calves was extracted from blood samples. These calves together with the corresponding embryos were genotyped with the Illumina Bovine 54k BeadChip. Call rates were recorded, correlations between embryo and calf genotypes calculated and breeding values estimated. The biopsy technique G1 resulted in the highest number of good quality transferable embryos G1 (1.37) vs. G2 (0.97) ($p<0.05$) in relation to the number of original embryos. However, better pregnancy rates were obtained by transferring 2 demi-embryos to one recipient (1 demi-embryo=28.6%; 2 demi-embryos=76.2%). Biopsy technique G2 resulted in 55.0% pregnancies. No discrepancies could be detected between gender, polled and hereditary defect status of born calves and corresponding embryos. The average call rate for the genotyped embryos was 0.922, ranging from 0.841 to 0.980. The call rate of the corresponding calves ranged from 0.998 to 0.999. The average concurrency of the obtained genotypes of embryos and calves was 98.7%, with an average correlation of 0.991. Gender, polledness and genotypes obtained from preimplanted embryos were consistent with genotypes obtained of the born calves. Therefore, our first results provide promising prospects for the optimized production line.

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Comprehensive proteomic analysis of *Gallus gallus* uterine fluid

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Keywords: *Gallus gallus,* proteomic analysis, uterine fluid.

Domestic hens are able to keep spermatozoa in their genital tract for long periods, and can so produce fertilized eggs for up to 3 weeks after one insemination. An extensive description of the avian uterine fluid proteome will help to provide the basis for a better understanding of a number of diseases and processes, including sperm survival but also female infertility and cell storage. Uterine fluid was collected (n=10) into a plastic tube placed at the entrance of the everted vagina 10h after oviposition. Bottom up proteomic approach using SDS-PAGE and nano LC-MS/MS (ultimate 3000 RSLC system coupled to LTQ Velos Orbitrap mass spectrometer) was performed with a high-low resolution MS strategy. Data were matched against NCBInr database using Mascot 2.3 and identifications were validated by the peptide and protein Prophet algorithm using Scaffold 4.0 software. Bioinformatics treatments of data set was carried out to refine annotation of proteins using NCBInr database, and to describe uterine fluid proteins using SecretomeP 2.0 and SignalP 4.1 tools, InterproScan software, and, Exocarta, KEGG and UniprotKB databases. Among a total of 922 proteins that were identified, 836 (91%) were identified in *Gallus gallus* databases, whereas 86 (9%) were identified in others species, indicating unknown chicken isoforms. Deepens analysis of cellular component revealed three categories of proteins. The secreted proteins (165) known to be secreted with a peptide signal or by an unconventional pathway, the exosomal proteins (644) which match against exosomal databases (Exocarta, UniprotKB, KEGG) and the last category refers to proteins which are not annotated as exosomal or secreted (113). Secreted proteins are composed of protease inhibitors (11), cytoskeletal and extracellular matrix proteins (22), enzymes (metabolic, proteases etc.) (49) and others proteins implied in calcification of eggshell (OC-17, OC-116). Exosomal proteins mainly consist in enzymes (metabolic, oxidoreductase) (225), chaperon proteins (HSPA8, HSP90AA1,...) (26) and proteins implied in MVB biogenesis (Alix, TSG101, Clathrin,…) (25). We have isolated exosomes and confirmed the presence of exosomal markers (CD63, HSPA8) by western blot in the avian uterine fluid. The presence of exosomal proteins in avian uterine fluid may represent a novel and exiting mechanism of cell-cell interactions, that may explain at least in part, the long term sperm survival. We believe that the thorough catalogue of proteins presented here can serve as a valuable reference for the study of sperm interaction with the female genital tract. Moreover, it could be an interesting tool for biomarkers discovery involved in fertility.

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Assessment of fertilizing ability of Iberian ibex (*Capra pyrenaica*) vitrified and frozen epididymal sperm by *in vitro* heterologous fertilization of bovine oocytes

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Keywords: bovine oocytes, epididymal sperm, goat, heterologous fertilization.

The aim of this study was to evaluate the fertilizing ability of vitrified and frozen Iberian ibex sperm by assessing heterologous IVF using bovine oocytes. Testes were obtained from mature ibexes that were legally hunted in the Tejeda and Almijara Game Reserve, in southern Spain. Epididymal spermatozoa were collected by the retrograde flushing method. Sperm from right epididymis was vitrified with TCG-6% egg yolk plus 100 mM sucrose while sperm from left epididymis was conventionally frozen with TCG-6% egg yolk and 5% glycerol. *In vitro* matured zona-intact bovine oocytes were subjected to heterologous IVF with vitrified-warmed (n=495) or frozen-thawed ibex sperm (n= 565) and homologous IVF (n=299). A non-fertilized group was included as control for parthenogenesis (n=81). For heterologous fertilization, sperm pool of three males was used for each treatment. Sperm-oocyte interactions were evaluated at 2.5 hours post-insemination (hpi) by the number of attached and bound spermatozoa whereas penetration and polyspermy were evaluated after 12 hpi. Presumptive zygotes were fixed and stained with Hoechst 33342 at 18, 20, 22, 24 and 26 hpi to assess pronuclear formation using a phase contrast and confocal microscopy. Besides, cleavage rate was evaluated in all groups at 24 hpi. Data obtained was analyzed using one way ANOVA (Sigma Stat, Jandel Scientific, San Rafael, CA) Results showed a higher number of bound and attached spermatozoa in both heterologous groups compared to homologous group ($P<0.001$). The homologous IVF group as expected, showed the highest percentage of pronuclear formation at 18 hpi (67.7±9.8%), significantly different to both heterologous groups (Frozen: 21.3±13.9%; Vitrified: 28.8±15.5%, $P<0.05$). Indeed, pronuclear formation was delayed in both heterologous groups with the highest percentage at 24 hpi (30.3 ± 15.1%) for frozen sperm and at 20 hpi (31.7 ± 21.5%) for vitrified sperm. In addition, cleavage rate was higher in homologous group compared with heterologous frozen and vitrified groups (76.1±15.9% *vs*. 31.3±27.2% and 45.1±24.4%, respectively, $P<0.05$). No differences were observed between heterologous vitrified and frozen sperm in all parameters evaluated. In conclusion, Iberian ibex epididymal sperm can be vitrified successfully, maintaining its fertilization ability in the same extend as frozen sperm. To our knowledge, this is the first report of successful epididymal sperm vitrification in a mammal species being capable of fertilization as a standard tool for genome conservation in threatened species.

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Cryopreservation of sheep embryos by slow freezing or vitrification with or without caffeic acid

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Keywords: antioxidant, caffeic acid, cryopreservation, plasminogen activator activity, sheep embryos, slow freezing, vitrification.

Reduced viability of embryos after cryopreservation has been associated with lipid peroxidation due to increased levels of free radicals. Thus the addition of antioxidants in the cryoprotectant solutions might be beneficial to embryo survival. Antioxidant caffeic acid, that has been shown to reduce the levels of lipid peroxidation markers in rat erythrocytes, was tested for its ability to improve the cryotolerence of sheep embryos under the two major methods used for embryo cryopreservation. Embryos were collected from 32 superovulated Lesvos ewes, on day 7 after the onset of oestrus, soon after slaughter, by uterine horns flushing and were evaluated under stereoscope. One hundred and thirteen freezable embryos (grades 1, 2), in the morula or in the blastocyst stage, were cryopreserved either by slow freezing (seeding at -6.5°C, 0.3°C/min to -35°C) or by vitrification. Unless differently specified, all chemicals were purchased from Sigma-Aldrich Co. (St Luis, MO, USA). Ethylene glycol, in a final concentration of 1.5M in ECM [Embryo Culture Medium = PBS+20%FCS (Biochrom AG, Berlin, Germany)], was used as cryoprotectant in slow freezing. A final concentration of 25% glycerol and 25% ethylene glycol in ECM was used for vitrification. In half of the cases in each method, 20μM of antioxidant caffeic acid was added in all the cryoprotectant solutions. After thawing / warming, the embryos were cultured in vitro, in SOF, for 72 hours and evaluated for development and hatching. Plasminogen activator activity (PAA), which has been linked to embryo development or degeneration, was determined spectrophotometrically in the media used during the removal of cryoprotectants and in vitro culture. Data was analysed using chi square test, t-test and regression analysis. Overall, 56.0% of the thawed / warmed embryos developed during in vitro culture. At the end of in vitro culture, 42.0% of all the incubated embryos were undergoing or had completed hatching; 42.3% after slow freezing and 41.7% after vitrification. Increased hatching ratio was observed in the embryos cryopreserved in the presence of caffeic acid (52.0% vs. 32.0%, $P<0.05$); this was apparent in both cryopreservation methods and the difference approached significance after slow freezing (53.8% vs. 30.8%, $P<0.10$) but not after vitrification (50.0% vs. 33.3%, $P<0.20$). At the end of in vitro culture, 47.0% of the embryos were degenerating; no statistically significant effect of cryopreservation method or the presence/absence of caffeic acid was observed. PAA in the culture medium at the end of in vitro culture was negatively associated with the ratio of degenerated embryos ($R2=0.465$, $P<0.05$). In conclusion, addition of antioxidant caffeic acid seems to improve cryotolerence of sheep embryos and its effect seems to be more prominent when slow freezing is applied.

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Vitrification of intact and splitted *in vitro* produced d7 bovine embryos

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Keywords: cattle, embryo splitting, embryos, IVP, vitrification.

In the present study, we compared the vitrification of intact and splitted *in vitro* produced bovine embryos with two vitrification methods: The CryoLogic Vitrification Method (CVM), CryoLogic® Australia and the Hollow Fiber Vitrification (HFV) Method (Matsunari et al. 2012). For IVP ovaries from slaughtered animals were used. Aspirated oocytes were *in vitro* matured (IVM) for 22 h, followed by *in vitro* fertilization (IVF) for 18 h. Presumptive zygotes were denuded and *in vitro* cultured (IVC) in SOF supplemented with 5% OCS. On D7, intact or splitted embryos were classified in grade I or II and vitrified, either by the CVM or by the HFV method. Embryos were loaded in 0.7-1.0 µl of vitrification solution. Vitrification and thawing procedures were performed as previously described (Saucedo et al., 30th Annual Meeting A.E.T.E., Dresden, Germany, 2014). After thawing, embryos were *in vitro* cultured until D12. Survival rate (judged by re-expansion) 24-48 h after thawing and hatching rate were recorded. Within the HFV method 273 (intact: HFV-) and 50 (splitted: HFV+), and within the CVM method 256 (intact: CVM-) and 312 (splitted: CVM+) embryos were cryopreserved. The percentage of lost embryos was lower in HFV- (0.7%) vs. HFV+ (9.0%) vs. CVM- (9.2%) vs. CVM+ (16.4%). The overall re-expansion rate was significantly higher with CVM than HFV (70.8 vs. 61.0%; $p>0.05$) and the highest results were obtained with blastocysts (73.6%) followed by early blastocysts (70.8%) and morulae (58.0%) ($p=0.004$, Kruskall-Wallis test). No significant differences were observed using intact or splitted embryos. Re-expansion rate of intact embryos resulted in 68.6% vs. 68.8% of splitted embryos ($p=0.835$; Mann-Whitney test). Survival of embryo regarding the time of culture between splitting and vitrification (3 or 20 h) showed a tendency to highest results after 20 h (63.0% vs. 72.1%; $p=0.807$, Mann-Whitney test). Demi-embryo survival and effect of embryo's stage on biopsy outcomes were evaluated. No significant difference was found among stages (59.0%, 67.2%, and 90.0%, for morulae, early blastocysts and blastocysts, respectively; $p=0.0568$ Kruskal-Wallis test) with regards to survival after splitting and biopsy. However, blastocysts leads to better survival after splitting and vitrification. In conclusion, both vitrification methods are suitable for intact or splitted bovine embryos, whereas the CVM seems to be more practical in handling.

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