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Cholesterol addition to immature and in vitro matured bovine oocytes before vitrification altered the expression of some genes important for early embryonic development

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Keywords: cryopreservation, PCR, cyclodextrin.

Cholesterol:phospholipid ratio and fatty acid composition are important factors for plasma membrane fluidity and permeability. Methyl-β-cyclodextrins charged with cholesterol can transfer the cholesterol into cellular membranes improving their cryotolerance. In the present study, we examined whether the exposure of immature (GV) and *in vitro*-matured (MII) bovine oocytes to 2mg/mL cholesterol-loaded methyl-β-cyclodextrins (CLC) (Horvath and Seidel, Theriogenology, 66:1026. 2006) for 30min before vitrification would improve their cryotolerance and embryo developmental competence. The expression of seven genes (DNMT3A, HSPA1A, MnSOD, BAX, CYP51, IGF2R, UBEA2) were analyzed in mRNA extracted from morulae obtained *in vitro* from GV and MII oocytes treated with CLC before vitrification using RT-PCR. Expression levels of the target genes were normalized to expression level of CHUK, which were expressed at similar levels in all oocyte samples and were stable under the conditions used. The effects of treatment and stage upon developmental competence were tested through a two-way analysis of variance (ANOVA), followed by a post-hoc Sidak's test for multiple comparisons. Effects on gene expression were evaluated through non-parametric Scheirer-Ray-Hare and Mann-Whitney tests (Scheirer et al., Biometrics 32:429.1976). The level of significance was set at $P < 0.05$. Vitrified oocytes showed lower cleavage rates and blastocyst yield than non-vitrified oocytes. No significant differences in terms of cleavage and blastocyst rates were observed between vitrified groups, regardless of CLC treatment and oocyte maturation status. No significant changes in relative mRNA abundance for HSPA1A, MnSOD and IGF2R genes were found in morulae from vitrified oocytes compared to non-vitrified oocytes, regardless of CLC treatment or oocyte maturation status. The expression of DNMT3 and BAX genes was significantly upregulated in morulae from vitrified GV oocytes, whereas gene expressions in CLC-treated vitrified GV oocytes remained similar ($P > 0.05$) to non-vitrified GV oocytes. Contrarily, the expression for CYP51 gene was significantly downregulated in morulae from vitrified GV oocytes compared to morulae from CLC-treated vitrified or non-vitrified oocytes. The expression of DNMT3A was significantly upregulated in morulae from MII vitrified oocytes, while no significant differences were observed in morulae from oocytes vitrified after CLC treatment. The expression of UBE2A was significantly downregulated in morulae from vitrified oocytes regardless of CLC-treatment and oocyte maturation status. In conclusion, treatment with cholesterol prior to vitrification of immature or *in vitro* matured bovine oocytes did not enhance embryo development. However, the treatment of oocytes with CLC before vitrification, altered the gene expression related to *lipid metabolism* (CYP51), apoptosis (BAX) and DNA methylation (*DNMT3A*) in bovine morulae.

This study was supported by the Spanish Ministry (AGL2013-46769) and Agriculture and Agri-Food Canada.

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MALDI-TOF mass spectrometry analysis of lipids in single bovine oocytes during IVM

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Keywords: oocyte, lipids, MALDI mass spectrometry.

Bovine oocyte is rich in intracellular lipids which are involved in membrane composition, intracellular signaling and energy storage. We have recently showed that level of neutral lipids containing in lipid droplets was diminished during oocyte *in vitro* maturation, IVM (Auclair et al. *Am J Physiol Endo Metab*, 2013,304(6): E599-613). We also reported that that Intact Cell Matrix-assisted laser desorption/ionization time of flight Mass Spectrometry (ICM-MS) analysis of lipid profiles of cumulus cells was able to discriminate immature and mature oocytes (Sanchez-Lazo et al. *Mol Endocrinol* 2014, 28(9):1502-1521). The objectives of this work were to adapt ICM-MS technology to single bovine oocytes and to compare lipid contents in the oocytes before and after IVM.
IVM was performed on bovine oocyte-cumulus complexes from 4-6 mm ovarian follicles in culture medium containing 10% of fetal bovine serum (MP Biomedicals, Illkirch, France), growth factors and gonadotropins. ICM-MS was performed on individual immature (n = 12) and mature (n = 12) oocytes, completely denuded from CC. Lipid spectral profiles (3000 shots per spectra) were acquired from each oocyte, cocrystallized with 2,5-dihydroxyacetophenone (DHAP) matrix, using an UltrafleXtreme MALDI-TOF/TOF instrument (Bruker) in positive reflector mode. M/z peaks were detected in the range of 160 to 1000 m/z and values of the normalized peak heights (NPH) were quantified using Progenesis MALDI™ (Nonlinear Dynamics). Coefficient of variation (CV %) was calculated for each m/z peak from 3 technical replicates using 20 immature oocytes. Multivariate Principal Component Analysis (PCA) and Student test were applied to NPH values for hunting lipid content variations between immature and mature oocytes. Lipids were extracted from follicles; several peaks were fragmented by high resolution MSMS top-down analysis using LTQ Velos Orbitrap operating in positive mode and annotated using LipidMaps.
A total of 266 distinct peaks ranging from m/z 163.27 to 951.62 were detected. Mean CV% of all the peaks was 32%. 72 peaks were differential between immature and mature oocytes (38 up- and 34 down-regulated during IVM, $P < 0.01$, fold change >2.0). Among them, several up-regulated peaks (2-68 fold increase during IVM) ranging from m/z 700 to m/z 815 were identified as phosphatidylcholines (32:0, 32:1, 33:1, 34:2, 36:2, etc) and sphingomyelins (36:1, 42:2). Among the down-regulated peaks, fatty acids C14:0 (16-fold decrease during IVM) and C17:0 (2-fold decrease) were annotated. Groups of immature and mature oocytes could be clearly discriminated by PCA.
In conclusion, lipid content significantly varied in the oocytes before or after IVM due to both changes of oocyte follicular environment to in vitro culture and to proper intracellular fatty metabolism (lipogenesis, lipolysis…) leading to structural modifications in the oocyte.

Financial support: Bovomega3 project funded by Val de Loire Region, France; thesis of P. Bertevello is funded by University do Estado do Rio Grande do Norte (Brazil).

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Sustainable regulation of metabolic performance of bovine embryos by L-Carnitine supplement and concurrent reduction of fatty acids

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Keywords: bovine embryos, L-Carnitine, fatty acids, IVP.

The increasing importance of in vitro produced (IVP) embryos in commercial cattle breeding programs demands improvement of embryo viability after cryopreservation. Positive effects of L-Carnitine (LC) supplement on lipid accumulation of IVP embryos, which is suggested to play a key role in viability after cryopreservation, has been shown previously.

In the present study we aimed to reveal the impact of LC supplementation and the concurrent reduction of fatty acids (FA) during in vitro culture on metabolic features, implicating the morphological as well as the molecular level.

In the control group presumptive zygotes were cultured in SOFaa media supplemented with BSA. In the three experimental groups either fatty acids were removed using BSA fatty acid free (FAF) or 2.5 mM L-Carnitine were added (BSA+LC) or removing of fatty acids and supplement of L-Carnitine were combined (FAF+LC). All embryos were cultured in groups of 50 in 400µl medium covered with mineral oil in 5% O_2 and 5% CO_2.

Concerning developmental rates, no significant differences were observed between the groups, therefore, the impact of either FA reduction or LC supplement could be excluded. In contrast, survival rates after slow-freezing (EG) and thawing of day 7 blastocysts show that LC positively affects re-expansion speed of the embryos. Significantly higher hatching rates were detected for embryos cultured in FAF+LC. Furthermore, lipid accumulation was quantitatively measured, by uptake and elution of Oil Red in day 7 blastocysts using a Microplate reader. Significantly lower amounts of lipid were detected in FAF and BSA+LC, implicating that either removal of FA or supplementation of LC reduce lipid content effectively. To understand molecular mechanisms affected by different culture conditions, a set of genes related to oxidative stress response (KEAP1 and SOD1) and lipid metabolism (AMPK, ACC and PGC1α) were determined using realtime PCR. Regarding KEAP1, playing multiple roles in the cascade of oxidative stress response, and SOD1, known to act as ROS scavenger, we detected significantly higher abundance in embryos cultured with FAF and FAF+LC compared to their counterparts cultured with BSA and BSA+LC. We suggest that not LC but the presence or absence of fatty acids influence oxidative stress response. A similar pattern was observed for AMPK being significantly higher expressed in embryos which were cultured in the absence of fatty acids. In contrast, the expression of ACC, known to play key a role in storage and synthesis of long chain fatty acids, and PGC1α, being responsible for mitochondrial activity, are affected by the presence of FA but also by supplement of LC in combination with removal of FA. In summary, we could unravel, the positive effect of LC and the concurrent removal of FA on cryosurvival, metabolism and gene expression.

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Impact of enrichment of unsaturated fatty acids during *in vitro* maturation and culture of bovine embryos on blastocyst rates

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Keywords: fatty acid, cleavage, blastocyst.

Bovine IVP embryos have significantly lower cryosurvival rates than in vivo embryos (Rizos et al., Mol. Reprod. Dev. 61:234-248, 2002). Preliminary data from our group showed that IVP blastocysts have a low degree of unsaturated fatty acids esterified to phosphatidylcholine (PC) when compared to in vivo blastocysts. Since the unsaturation degree of PC determines membrane fluidity, this difference may explain the lower cryoresistance of IVP embryos. The main objective of our study is to improve cryosurvival rates of IVP embryos by increasing the unsaturation degree of PC. In this abstract, we describe whether the addition of unsaturated fatty acids (FA) to in vitro maturation (IVM) or in vitro culture (IVC) media affects embryo development. Oleic acid (OA) and linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid; LA) were complexed to FA free BSA and added to FA free IVM or IVC media. In experiment 1, cumulus oocyte complexes (COCs) were exposed to either 0 µM FA (control), 700 µM OA, 700 µM LA or a combination of 350 µM LA and 350 µM OA during the entire IVM period before routine embryo culture (levels of FA based on; Bender et al., Reproduction 139;1047-1055, 2010). In experiment 2, presumptive zygotes were exposed to either control BSA (final concentration 350 µM FA), 350 µM OA, 350 µM LA or a combination of 175 µM LA and 175 µM OA during the entire IVC period. For all experimental conditions 3-4 replicas of ± 115 COCs each were tested. Statistical analysis was performed using a univariate general linear model with day 5 (post-fertilization) cleavage rate and day 8 blastocyst rate as dependent variables in IBM SPSS Statistics 22. In experiment 1, none of the FA-BSA additions to the IVM medium had an effect ($P > 0.27$) on cleavage rates (control 84.3 ± 3.1%; OA 85.2 ± 2.8%; LA 79.8 ± 3.2%; LA/OA 82.2 ± 3.2%) nor blastocyst rates (control 39.4 ± 3.3%; OA 39.7 ± 3.0%; LA 33.8 ± 3.4%; LA/OA 34.1 ± 3.4%). In experiment 2, none of the FA-BSA additions to the IVC medium had an effect on cleavage rate ($P > 0.17$) (control 81.9 ± 1.4%; OA 83.4 ± 1.4%; LA 84.7 ± 1.4%; LA/OA 84.5 ± 1.4%). Addition of 350 µM OA did result in similar blastocyst rates ($P > 0.28$) as observed in the control groups (control 34.2 ± 1.3%; OA 31.7 ± 1.3%). However, addition of 350 µM LA significantly reduced blastocyst rates (LA 26.2 ± 1.3% ($P = 0.004$); while addition of equimolar amounts of LA and OA (both 175 µM) inhibited the adverse LA effect (blastocyst rate 31.5 ± 1.3% was indifferent to control ($P > 0.3$) and significantly higher than LA only ($P = 0.006$). In conclusion, addition of FA-BSA to the IVM medium did not affect embryo developmental competence. In contrast, addition of LA to the IVC medium inhibited blastocyst formation significantly while the presence of OA did not affect blastocyst rates and restores early embryo development competence in presence of LA. Future studies include lipid analysis and measurement of cryosurvival parameters of embryos matured and cultured under the different FA conditions.

Dr. C.H.A. van de Lest helped us with the statistic analyses.

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Viability of porcine vitrified morulae and blastocysts stored in a dry-shipper for 3 days

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Keywords: Vitrification, dry shipper, porcine embryos.

In the last decade, vitrification has emerged as an efficient tool for porcine embryo cryopreservation. Vitrified samples are highly sensitive to temperature oscillations, because embryos are vitrified in very small volumes that could suffer devitrification. To date, the only efficient way to transport vitrified embryos was the liquid nitrogen (LN) tanks. However, at present, the air movement of devices containing LN is subjected to a strict regulation since LN is considered as hazardous material. Recently, dry vapour shipper (DS) containers have been developed for the safe transport of biological samples at cryogenic temperatures (-150º). These devices guarantee a stable temperature for several days, which may allow the long-distance transport of vitrified embryos. The aims of this study was to assess the efficacy of a DS (ST reproductive technologies LLC, Navasota, USA) to maintain the viability of vitrified porcine embryos for a 3 days storage period compared to the routine storage in LN. For that, donor sows were subjected to a laparotomy six days after estrus. Embryos were collected by flushing the tip of each uterine horn with Tyrode's lactate (TL)-HEPES-PVA (TL-HEPES) medium. Only embryos at the morula and blastocyst stages showing good or excellent morphology according to the criteria determined by the IETS were used in the experiment. Vitrification and warming were performed with the superfine open pulled straw method using TL-HEPES as basic medium and ethylene-glycol and dimethyl sulfoxide as cryoprotectants. After vitrification, SOPS straws containing the embryos were stored in a LN tank for one month. Then, the straws were transferred from the LN tank to a DS (DS group) or to other LN tank (control group) for additional three days. After warming, DS (N = 47) and control (N = 46) embryos were cultured for 24 h to assess embryo survival (ratio of viable blastocysts at the end of culture to the total number of embryos cultured) and hatching rates. The results were analysed using Fisher's exact test. In both groups, the embryos vitrified at the morula stage (N = 48) displayed the same survival rates (95.8%) and similar embryo development stage at the end of the culture (34.6% and 65.4% of DS embryos and 43.4% and 56.6% of control embryos were early-full and expanded-hatching blastocysts, respectively). When the embryos were vitrified at the blastocyst stage (N = 45), there were also no differences between groups in the survival rates (95.6 vs. 100%) and embryo development (100% of the embryos were at the perihatching or hatching stage at the end of de culture in both groups). The present study shows the efficacy of the DS for the storage of vitrified porcine morulae and blastocysts for a minimum of 3 days, which allows the international air transport of porcine embryos safely.

Supported by MINECO-FEDER (AGL201569735-R) and Séneca (19892/GERM/15).

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Comparison of the effects of slow freezing and vitrification on *in vitro* embryo quality in horse and donkey

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Keywords: Equine, cryopreservation, embryo.

The objective was to assess the effects of two methods of cryopreservation on the quality of Day 6.5-7.5 embryos (<300 mm) from mares and jennies. In group 1 (sf; n = 5 horse embryos and n = 4 donkey embryos), embryos were incubated at room temperature for 10 min in 1.5M ethylene glycol and then were frozen in 0.25ml plastic straws using Cryologic CL-3300 controlled-rate freezer. In group 2 (vf = 5 horse embryo and n = 4 donkey embryos), embryos were vitrified by exposure to VS1 for 5 min, moved to VS2, and then transferred into VS3 (Eldridge-Panuska et al., 2005). The embryo was loaded in a 3µl drop of VS3, was placed on the Fibreplug device and it was put in contact with precooled CMV block surface (CryoLogic, Pty Ltd, Victoria, Australia) for some second. After storage, the frozen embryos were thawed for 30 sec in a 37ºC water bath. Vitrified embryos were warmed by immersion for 10 min in holding medium containing 0.25M galactose at 37ºC. After thawing/warming, embryos were morphologically assessed and then stained with DAPI-TUNEL-Phalloidin stain. Embryos were assessed by confocal laser-scanning microscopy (LSM 710, Carl Zeiss Jena, Germany). GLM repeated measures analysis was used to analyze the embryo quality at recovery and after thawing/warming, and ANOVA was used to evaluate the effect of the cryopreservation methods on the percentage of dead cells, fragmented/condensed nuclei and apoptotic nuclei. The percentage of embryos with different cytoskeleton quality was analyzed by c^2 test. A significant decrease in embryo quality was observed after cryopreservation in all the groups ($P < 0.05$). The percentage of death cells after slow freezing was significantly lower than after vitrification in horse (5.0 ± 2.2 vs 9.8 ± 1.6, $P < 0.01$), but not in donkey (4.6 ± 3.2 vs 3.0 ± 0.1; n.s.). The percentage of apoptotic cells was significantly different in relation to cryopreservation methods and species ($P = 0.002$ and 0.022, respectively). No significant differences between groups were detected for the percentage of DNA fragmented or apoptotic cells. In relation with the quality of actin cytoskeleton of embryos, no differences between species or methods were detected. In horse embryos, when slow freezing was used, the cytoskeleton showed grade I in 40% of embryos and the remaining showed grade II. Embryos vitrified over solid surface and with Fibreplug had grade I in 20% of embryos and grade II in the other 80%. Embryos recovered from mares were more susceptible to vitrification than to slow freezing, since more cellular injuries were induced. In contrast, vitrification of donkey embryos induced lower cellular death and apoptosis than slow freezing, and it could suggest that vitrification is a good method for embryo cryopreservation in donkeys, which were more resistant to this method than horse embryos.

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Supported by INIA-FEDER RZ2008-0002.

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The influence of DNA stabilizing buffer on the results of genomic bovine embryo analysis

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Keywords: embryo biopsy, DNA conservation, TE buffer.

Nowadays breeding programs involve the biopsy of preimplantation bovine embryos to determine sex (S), polled status (PS), hereditary defects (HD) and early genetic selection. Those techniques are more cost effective the higher the success rate of analyzed samples. To minimize the loss of information due to DNA degradation the obtained cells must be successfully stored in tubes prior to whole genome amplification.
The objective of this study was to analyze the use of a DNA stabilizing buffer on the successful outcome of S, PS, HD determination and on the call rate after chip SNP analysis.
Embryos were obtained on day 7 after insemination by superovulation of German Simmental animals with a standard protocol. Immediately after recovery, embryos were biopsied by a single operator under a mobile stereo microscope (Olympus, Japan) at 50x magnification and a steel blade mounted on a blade holder (Bausch & Lomb, Germany) attached to a micromanipulator (Eppendorf, Germany). Two different biopsy media (BM) were used, (1: PBS from IMV, France or 2: 0.9% NaCl solution with 1.5% PVP). Removed cells were brought to reaction tubes by pipetting them with 0.5 µl medium in either the empty tube or in 2.5 µl TE buffer (TE buffer, 10 mM Tris, 1 mM EDTA, pH 8.0). Biopsied cells, approximately 10, were immediately used after biopsy for whole genome amplification (Repli-g Mini Kit, Qiagen, Germany) followed by PCR analysis of S and PS. HD were analyzed using a 5'-exonuclease assay. Embryos were genotyped with the Illumina Bovine 54k BeadChip. Call rates were recorded. Differences in success (%) and call rates were analyzed by proc GLM, SAS (fixed factors: BM (1+2), TE (yes/no), developmental stage of embryo, morulae (4), early blastocyst (5), blastocyst (6), and expanded blastocyst (7).
In total 503 embryos were analyzed for S, PS and HD. From the total number of embryos 101 Embryos were SNP analyzed and call rates obtained. The BM had no influence on the success rate of positive analyzed S (90.7% vs. 86.0%), PS (89.4% vs. 86.9%) or HD (90.0% vs. 82.8%), $P > 0.10$. The stage of embryo had also no influence on the success rate of S (4= 90.1%, 5= 85.7%, 6= 87.9%, 7= 85.0%, $P > 0.10$), PS (4= 89.8%, 5= 85.2%, 6= 86.5, 7= 95.0%; $P > 0.10$), HD (4= 89.8%, 5= 85.2%, 6= 86.5%, 7= 86.7%, $P > 0.10$) analysis and call rate (4= 0.8939, 5= 0.8893, 6= 0.9211, 7= 0.9224, $P > 0.10$). Highly significant differences showed the use of TE buffer vs. no TE buffer for call rate (0.9137 vs. 0.8396, $P < 0.0001$), S (91.4% vs. 78.0%, $P < 0.0001$) and PS (90.9% vs. 79.7%, $P < 0.0001$) and HD (89.3% vs. 74.3%, $P < 0.0001$). The biopsy medium and the stage of embryo had no effect on the success rate for the analysis of S, PS and HD. The use of a DNA stabilizing TE buffer improved results by 10-15%. Therefore, we advise the use of TE buffer for storing small embryo cell samples to optimize analysis.

Funded by the Bavarian Research Foundation (AZ-1031-12).

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JC-1 dye is a valuable indicator of embryo health in rabbits

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Keywords: Mitochondrial metabolism, Embryo cryopreservation.

One of the main challenges in embryo biotechnology research is to develop *in vitro* evaluation methods of embryo quality to detect early alterations correlated with embryo development competence. JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) is a lipophilic cationic dye, which accumulates within mitochondria according to its mitochondrial membrane potential (MMP) emitting different fluorescent properties. High MMP mitochondria accumulate more cationic dye (J aggregates) and exhibits red fluorescence, while low MMP mitochondria accumulate J monomers, showing green fluorescence. Disruption of MMP has been associated with metabolic stress and early cellular apoptosis.

Our aim was to adapt a JC-1 staining method to fresh and cryopreserved rabbit embryos in order to evaluate their quality. To this end, embryos (n = 64) were collected at the morula stage, and were randomly divided into two groups: fresh (n = 39) and slow frozen (n = 25; DMSO, 1.5 M) embryos. All embryos were analyzed at the blastocyst stage, according to morphological quality and classified as normal (inner cell mass and trophectoderm quality considered as good or fair) or damaged embryos (delayed, fragmented (> 20%), poor quality inner cell mass and trophectoderm, and smaller blastocysts were considered as damaged). Embryos were pretreated with pronase (Roche, Meylan, France), stained with JC-1 (1.5 μM; Invitrogen, Life Technologies, Eugen, Oregon, USA) for 75 min (38.5°C, 5 % CO2) and observed under an epifluorescence microscope. CCCP (Sigma-Aldrich, Saint Quentin Fallavier, France), a MMP disruptor, was used as a control to confirm the JC-1 sensitivity to changes in MMP. The staining intensity (pixel) was determined in two randomly defined areas on each embryo. In order to dismiss the potential effects of the cryopreservation process on stain uptake, only the red/green ratio was analyzed using ImageJ software. Significant differences were found between fresh (R = 3.55 ± 0.94) and normal cryopreserved embryos (R = 2.55 ± 0.78; P < 0.01), as well as between normal and damaged (R = 1.03 ± 0.50; P < 0.05) cryopreserved embryos.

We conclude embryo' morphological defects are associated with MMP disruption, and cryopreservation seems to impair the mitochondrial metabolism even in absence of identifiable alterations of the embryo. This study is the first to describe a JC-1 staining protocol for rabbit embryo evaluation, showing it can be used as a valuable indicator of embryo' health and functionality for this species.

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Rabbit embryo vitrification without animal products

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Keywords: Animal derived products, Cryopreservation methods, Bovine serum albumin.

Embryo cryopreservation often requires the use of animal derived products (such as bovine serum albumin (BSA) and fetal calf serum (FCS)), which represent a sanitary risk, and contain undefined material that can considerably vary between batches, altering its thermodynamic properties. CRYO3 (Stem-Alpha, Saint-Genis-l'Argentière France) is a chemically defined substitute without animal products, created for mononuclear cell cryopreservation.
Recently, our research team successfully slow freezed rabbit embryos with a buffer medium composed of D-PBS (Dutscher, Brumath, France) supplemented with 20% of CRYO3 and 1.5M DMSO (Bruyère P et al. Plos One 8(8): e71547, 2013).
Our objective was to compare three rabbit embryo vitrification buffer media: IMV holding medium (IMV, L' Aigle, France): a commercial medium containing BSA (G1); D-PBS supplemented with 20% of CRYO3 (G2); and a CRYO3 medium (G3). All the media contained the same cryoprotectant composition.
Rabbit New Zealand does (n = 12) were submitted to a superovulation treatment and collected embryos (n = 231) were randomly divided into three groups. After equilibration, embryos were exposed for 30 sec to a vitrification solution of G1 / G2 / G3 medium, containing 20 % Me2SO and 20 % EG, before being loaded to the hook at the end of a custom designed fibre called a Fibreplug™ (CVM Kit, Cryologic, Victoria, Australia) and vitrified by solid surface vitrification. Thawing was performed by immersing the end of the Fibreplug™ directly into a thawing solution (0.5 M sucrose, respectively), for 5 min, followed by three successive dilution baths. Embryos were cultured to the hatching stage in M199 medium (G1: 52, G2: 37 and G3: 71 embryos), supplemented with 10 % FCS (38.5°C, 5 % CO2). The survival rate after thawing (embryos with <20 % or no fragmentation, per vitrified embryos), the blastocyst formation rate, and the hatching rate (per survived), were evaluated, and analyzed with Chi-square test; Delayed, fragmented (>20%) and smaller blastocysts were considered as damaged. The survival rate (G1: 87 %, G2: 81 % and G3: 96 %)) was significantly superior in the CRYO3 group ($P < 0.05$). No significant difference was observed regarding the blastocyst formation rate at 48 h (G1: 77 %, G2: 65 % and G3: 66 %) or the hatching rate (G1: 35 %, G2: 19 % and G3: 24 %).
In conclusion, CRYO3 can be used as a chemically defined substitute for animal-based products in rabbit embryo vitrification solutions, reducing the solutions' variability and the sanitary risk inherent to animal derived products.

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Ultrasound Monitoring of reproductive organs in Angora bucks

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Keywords: accessory glands, doppler ultrasound, transrectal massage.

Objectives: The current study was design to monitor and create an evaluation method of reproductive organs (testis, epididymis and accessory glands) in clinically healthy Angora buck through ultrasound (US).
Material and Methods: Sixteen clinically healthy adult Angora bucks (ranging 1-4 year of age) were used. Classical breeding soundness examination was performed for each buck. Body condition score (BCS), testicular dimensions (Length, width and thickness) and scrotal circumference were measured with caliper, and as well as testis, epididymis and spermatic cord ultrasound (US) monitoring were assessed. Monitoring were performed longitudinal and transversal sections to evaluate the echo texture of the testicular parenchyma (Echo-TTP) including the mediastinum, tail epididymis (Echo-TEP) and colour doppler of vascular cone (EchoDop-VC) of each animal. The US of accessory glands was done in standing position using endocavitary linear probe (MyLabVetTM One, ESAOTE S.p.A., Genova, 10 MhZ probe frequency). Vesicular gland (Echo-VG) and bulbourethral gland (Echo-BG) were monitored with the landmark of urinary bladder. Testicular parenchyma was scored (0-3) according to echogenicity of the testis tissue. The tail of epididymis was evaluated for US appearance in relation with testicular parenchyma. Vascular cone monitoring was performed according to blood flow rate with using colour doppler US. First and foremost, sperm collection was carried out with transrectal ampullar massage. Spermatological parameters of volume, colour, viscosity, mass activity, motility and concentration were evaluated.
Results: High correlations were found among sperm volume and EchoDop-VC (R^2 = 0,6), sperm concentration with Echo-VG (R^2 = 0,9) and Echo-BG (R^2 = 0,8). Sperm motility and viscosity were also highly correlated according to Echo-VG (R^2 = 0,7). Echogenicity was increased progressively with age and with low body condition score presenting the testicular parenchyma granular none-homogeneous echogenic pattern. The echogenicity of the epididymis was although homogeneous and visual isoechoic with testis parenchyma.
Conclusion: Doppler US and echo-texture of external reproductive organs and accessory glands gives us valuable information on sperm production and related parameters. In addition, according to these results early detection of reproductive pathological conditions can be optimized with Doppler US in Angora goat.

Project was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) and Ankara University (Project No:213O034).