

## PRELIMINARY ATTEMPTS TO VITRIFY SHELLFISH OOCYTES

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In contrast to the small number of oocytes available for cryopreservation in agricultural species, shellfish produce millions of oocytes each spawning. Hundreds of thousands of offspring can therefore be produced from a given female and genetic gains can be made easily through maternal as well as paternal lines. Cryopreservation of Pacific oyster (*Crassostrea gigas*) and Greenshell mussel (*Perna canaliculus*) oocytes has been reported using slow cooling and the oyster protocol, although giving variable post-thaw fertilization and subsequent larval development, can be applied in selective breeding. The mussel protocol can not as subsequent larval development is poor (2% at best). Detailed experiments have been conducted to determine the damaging steps in the cryopreservation process. Oyster oocytes tolerate CPA addition but show marked developmental losses around -10°C and between this temperature and -35°C. Mussel oocytes are damaged by CPA addition and by cooling to -10°C. Vitrification offers an alternative approach; particularly for mussel oocytes which appear to be more susceptible to chilling injury. This paper reports preliminary attempts to vitrify shellfish oocytes. Ethylene glycol, dimethyl sulphoxide, glycerol, propylene glycol and butylene glycol were evaluated at varying concentrations alone and in combination in 0.25 ml straws and open pulled straws for their ability to vitrify and remain free of ice during cooling and warming. Subsequent experiments evaluated the toxicity of these CPAs, various methods of addition and removal and vitrification. Successful fertilization was achieved indicating tolerance to the levels of CPAs for short periods. However, no oocytes survived vitrification. Research is on-going.

## THE EFFECT OF EQUILIBRATION TIME ON SURVIVAL AND DEVELOPMENT RATES OF MOUSE BLASTOCYST STAGE EMBRYOS VITRIFIED IN SOLID SURFACE (SSV)

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The objective of this study was to investigate the effect of equilibration time on survivability and development rate of mouse blastocyst vitrified in Solid Surface Vitrification (SSV) method. In this study, mouse blastocyst were equilibrated by using two different time period for 10 min and 13 min in M2 medium containing 0.4% EG, vitrified in M2 medium containing 35% (v:v) EG, 5% PVP40, 0.4M trehalose for 20 to 30 sec. Than embryos were dropped on precooled to -150°C metal surface. After vitrifying drops, they stored in LN2 and warming was performed by containing 0.3M trehalose solution at 37°C for 3 min. Warmed embryos were washed in M2 medium and they were cultured in SAGE-Blastocyst Medium for 1 day. After the culture period, development rates of embryos were observed and they stained with Hoechst33342 for counting total cell number. In first group 34 of 49(69) blastocyst were survive at post warming period and 23 of 49(47) expanded blastocyst were continued to develop post culture period. In second group 47 of 51(92) blastocyst were survive at post warming period and 31 of 51(61) expanded blastocyst were continued to develop in post culture period. The total cell number of two experimental and control groups were 36,47 and 62 respectively. In conclusion, vitrification of mouse blastocyst stage embryos by SSV can result in high rates of in vitro development to expanded and hatched blastocyst stage and total cell number for 10 min equilibration time.

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## THE EFFECT OF VITRIFICATION ON BOVINE EAR AND MUSCLE TISSUES EXTIRPATED AFTER DEATH AND MAINTAINED AT DIFFERENT TIME PERIODS

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Cryopreservation of livestock ear and skin tissue can be applied in cloning, transplantation and endangered biodiversity conservation ex situ. In this study we investigated the effect of stored postmortem cartilage and muscle tissues at +4 °C for 5,24,48,72,96 and 216 hours on vitrification and post thawed vitrified and fresh tissues preparation of primarily tissue on culture obtained somatic cells. Postmortem tissues were maintained in PBS+1% PSA. Vitrification procedure were performed by exposing 1mm<sup>3</sup> tissues to 3,58M ethyleneglycol, 2.82M DMSO in PBS supplemented with FCS%20. Then tissues were plunged into LN2 after transferring to 0,5 mm payets. Warming and dilution of tissues from cryoprotectants was performed by washing 0,5M and 0,25 M Sucrose for 5 min respectively. Vitrified and fresh tissues were seeded in a35mm petri dishes containing DMEM/F12 supplemented with 20% (v/v) FCS and incubated 5% CO2 in air at 95% relative humidity and at 37 °C. The culture was started to changed after 7 days and changed every 2 days for a25 days maximum. After obtained the cells from vitrified and fresh tissues; H-E staining, population doubling time and MTT analyzes were done. As a result; we obtained the healthy cell population from all vitrified tissues but the cartilage cells were more quickly grown than the fibroblast cells which obtained from muscle tissues. After warming of the tissues; the mitotic activity of derived cells were decreased than the cells from fresh tissues. However, there is no significant differences between the all experimental groups. The producing cells established until 216 hours from fresh cartilage tissues that stored at +4°C. The similar results were found after warming the same tissue samples. According to these results; it is possible to obtain cell lines from the vitrified and fresh tissues until at +4 °C and 216 hours.

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## SEMEN CRYOPRESERVATION OF THE ARABIAN SAND GAZELLE MALES (GAZELLA SUBGUTTUROSA MARICA) USING TRILADYL AND TRIS DILUENTS

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The cryopreservation of sperm from endangered animals still needs more work to achieve higher successful rate. The aim of this study to investigate the proper methods of semen dilution and the Cryopreservation efficiency of the semen collected from the Arabia Sand Gazelle Males.

Semen was collected monthly for one year from 10 adult sand gazelles using electro-ejaculation method, at King Khalid Wildlife Research Center in Riyadh Saudi Arabia. For Cryopreservation, two semen diluents were used, commercial diluents Triladyl (Minitub, Germany) the second was Tris- citrate, laboratory prepared. Tris-citrate-based semen extender contains: Tris 3.63gm, citrate 1.99 gm, glycerol 5 ml, egg -yolk 10 ml, and fructose 0.5gm, per 100 ml, with osmolarity of (450mosm/kg) and pH 7. Diluted semen was divided in 0.25ml and Cryopreserved using Freeze control, CL-8000. USA program 7).

The over all mean of semen characteristics before freezing shows that the highest semen volume collection was recorded during October and November ( 0.49±0.12 ml), compared to the lowest during August (0.16± 0.02 ml). The overall mean semen volume was (0.304 ±0.11 ml), sperm concentration (816.3± 26.7x 10<sup>6</sup>), sperm motility (68.4±2.2%) and the live sperm was (73.08±2.05%). When the average of individual motility before freezing was compared with that after thawing, it was found that a

significant number of spermatozoa ( $P < 0.01$ ) was adversely affected by freezing. A significant difference ( $P < 0.01$ ) was also found between individual motility post-thawing and the two diluents Triladyl-diluted ( $35.8 \pm 2.1\%$ ) and Tris ( $25.8 \pm 3.07$ ).

It is concluded for Cryopreservation, the best results were obtained when Triladyl extender was used rather than Tris diluents.

#### **CRYOPRESERVATION OF HUMAN OVARIAN TISSUE: EFFECT OF DIFFERENT VITRIFICATION SOLUTIONS AND PROCEDURES**

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The aim of this study was to study different vitrification protocols to cryopreserve human ovarian tissue. To this end, the experiment was divided into three parts: (1) assessment of the toxicity of vitrification solutions (VS) on preantral follicles, (2) ability of VS to vitrify in contact with liquid nitrogen, and (3) capacity of vitrification protocols to preserve follicular morphology. Preantral follicle morphology and integrity were assessed by histology. Cellular proliferation and apoptosis were evaluated by immunostaining. In the first experiment, all solutions (VS1: 2M DMSO + 2M PROH + 0.2M sucrose + 12% FBS; VS2: 15% EG + 15% DMSO + 6% FBS; VS3: 38% EG + 0.5M trehalose + 6% FBS) significantly affected preantral follicles compared to fresh controls (percentage of normal follicles: control: 100%; VS1: 92%; VS2: 88%; VS3: 94%). Since only VS3 was shown to completely vitrify in the second experiment, it was used to cryopreserve tissue using three different systems: plastic straws, VS droplets and solid-surface vitrification (SSV). In all vitrification systems, the percentage of normal follicles detected was lower than in controls (percentage of normal follicles: control - 100%; straw - 15%; VS droplet - 47%; SSV - 21%). However, of the three protocols, the VS droplet approach yielded a significantly higher proportion of normal follicles.

In conclusion, high concentrations of cryoprotectants in VS affect follicular morphology and the tested vitrification procedures do not appear to properly preserve follicles, since more than 50% of the follicle population was not able to maintain its morphological integrity.

#### **COMPARISON OF TWO CRYOPRESERVATION METHODS FOR HUMAN EMBRYONIC STEM CELLS (hESC)**

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**Introduction:** Effective freezing and thawing techniques for hESC would enable the efficient preservation of stocks and for the establishment of hESC banks. The aim of this study is to compare two different methods for hESC cryopreservation.

**Material and Methods:** Two different cryopreservation methods were compared for two different lines, ES [2] and ES [6] (Barcelona Node, Spanish Stem Cell Bank). A slow freezing protocol (ultraslow protocol, USP) was compared with the standard method used for hESC freezing. Both methods consist in slow freezing and rapid thawing, with the use of a freezing box in a  $-80^{\circ}\text{C}$  freezer (cooling rate:  $-1^{\circ}\text{C}/\text{min}$ ) for the standard method and a programmable freezer (cooling rate of  $-0.5^{\circ}\text{C}/\text{min}$ ) for the USP.

The survival rate after thawing and the number of days needed for the first passage were compared between the two groups. Chromosomal stability and pluripotency were tested after thawing.

**Results:** A total of 385 hES colonies from the two hESC lines were frozen with each method. The survival rate was 26% for the USP and 5.1% for the standard method ( $P < 0.05$ ). The

number of days needed for the first passage was 8.2 for USP and 13.3 for the standard method ( $P < 0.05$ ). Morphological assessment of differentiation was performed, with better results using USP.

The cells kept normal karyotype and expression of the pluripotency markers after thawing.

**Conclusion:** The USP method is the preferred method for hESC freezing due to higher survival rate, with no changes either in karyotype and pluripotency markers.

#### **RECOVERY OF CUMULUS-OOCYTE COMPLEXES (COCs) AND INVESTIGATION ON OVIDUCTAL EPITHELIAL CELLS, PROTEIN SECRETIONS FROM OVIDUCT AND OVARY ON PORCINE ESTROUS CYCLE**

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The objectives of the project were studied the recovery of cumulus-oocyte complexes (COCs) and investigation on oviductal epithelial cells, protein secretions from oviduct and ovary on porcine estrous cycle. One hundred and ten ovaries of Large White reproductive pigs were gently aspirated follicular contents of healthy follicles with diameter about 2-6 mm. We found that COCs were round shape surrounded with clear zona pellucida and multi-layers of cumulus cells. They were ranging between 100-150  $\mu\text{m}$  in diameter. The number of COCs in follicular and luteal phase was 557 and 323 respectively. They can be classified into 5 types; intact-, multi-, partial-, and expanded-cumulus cell layers. While, the morphology of porcine ampulla and isthmus epithelial cells in estrous cycle showed 2 types of simple columnar ciliated cells (7-10  $\mu\text{m}$  in diameter) and round shaped non-ciliated cells (4-5  $\mu\text{m}$  in diameter). Moreover, the fluids from ampulla and isthmus part of oviducts and follicular fluids from follicles were detected the protein bands by SDS-PAGE. Utilization of LC/MS/MS technique for identifying selected protein bands from ampulla fluids in follicular phase found that the  $>220$  kDa and 240 kDa protein was probably trypsin or protease. For the ampulla fluids in luteal phase, the protein sized 95 kDa was found to be a 90 kDa heat shock protein while the protein sized about 105 kDa may be glycogen phosphorylase, protein kinase, or oviductal glycoprotein. The follicular fluid proteins in follicular phase at sized about 27 kDa, 45 kDa, 140 kDa, and  $>220$  kDa were found to be immunoglobulin, haptoglobin, complement component 3, apolipoprotein A-IV, ceruloplasmin, hemopexin and fibronectin respectively whereas the follicular fluid proteins in luteal phase at size of  $>220$  kDa was alpha-2-macroglobulin.

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#### **OXIDATIVE STRESS AFTER VITRIFICATION OF YOUNG AND AGED MOUSE OOCYTES**

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The regulation of intracellular redox potential is a crucial determinant of the biological competence of mammalian oocytes. Oxidative stress, indeed, is considered the main factor responsible for reduced oocyte quality related to aging processes. The aim of this study was to evaluate whether vitrification induced increased ROS production in mouse oocytes by taking into account the effects of oocyte ageing processes. To this purpose metaphase II oocytes were vitrified soon after their retrieval from young (4-8 weeks) and reproductively old (48-52 weeks) mice. Parts of the oocytes from young animals were cryopreserved after 6-hr incubation (in vitro aged oocytes). For the assessment of ROS levels we used a fluorescent dye, the 5-(and 6)-carboxy-2'-7'-dichlorofluorescein diacetate (carboxy-H2DCF). Semiquantitative analysis of fluorescence intensity revealed

that fresh "in vitro aged" and old oocytes exhibited increased ROS levels when compared to fresh young cells. Vitrification caused a significant increase of ROS production in young oocytes whereas "in vitro aged" and old oocytes showed levels of fluorescence intensity similar to their relative fresh controls. We also observed that in vitrified young cells ROS reached a level similar to that monitored in old and "in vitro aged" oocytes. Present results suggest that that vitrification negatively influences intracellular redox homeostasis in young oocytes and support the hypothesis that oxidative stress may negatively impact the developmental competence of vitrified oocytes.

#### **THE ASSESSMENT OF CRYOPRESERVATION CONDITIONS FOR HUMAN UMBILICAL CORD STROMAL STEM CELLS TOWARDS A POTENTIAL USE FOR STEM CELL BANKING**

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Human umbilical cord stromal stem cells are now considered as a remarkable and promising stem cell source to be used in cellular therapies. While no graft rejection has been reported in the recipient organism even in xenotransplantation studies, they were experimentally shown to attenuate tumor cell growth and gene transfers. In present study, we demonstrate a reliable and efficient cryopreservation method of HUCSCs resulting in the highest cell survival rate reported so far. Conventional, multi-step slow freezing (MSSF), and vitrification methods were comparatively tested using permeant (DMSO, ethylene glycol) and non-permeant (trehalose, sucrose, HES, human serum albumin) cryoprotectant agents (CPAs). After determining the crystallization point for each solution, latent heat evolution were suppressed during freezing and cooling (-40°C at 1°C/min or 0.3°C/min then cooling to -80°C at 5°C/min). The efficiency of the cryopreservation techniques used was determined by cell viability assays, the expression of cell surface markers and cytoskeletal proteins. The cell survival rate was found highest by MSSF with sucrose+DMSO at 1°C/min freezing rate. In this group no significant difference was noted before and after the cryopreservation in cell morphology, cytokeratin, vimentin, desmin and alpha-smooth muscle actin profiles and the expressions of CD105, CD90 and CD29. Second highest cell survival ration was obtained using 6% HES+DMSO group at 0.3 °C/min freezing rate. Together, our results indicated that MSSF favors to the other freezing protocols with an addition of sucrose or HES depending on the freezing rate used. (This study was supported by The Turkish Ministry of Industry STZ 00139.2007/2 project).

#### **INTEREST OF THE GLUTAMINE AND LDL (LOW DENSITY LIPOPROTEIN) IN FREEZING CANINE SPERM**

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Introduction: Various studies have demonstrated the cryoprotective action of glutamine (Glut). This preliminary study aims to determine the ideal concentration of glutamine when combined with 6% LDL to improve the cryopreservation of canine semen.

M&M: Exp n°1: 20 ejaculates were collected from 6 dogs (Beagle, Golden Retriever) aged from 3 to 6 years. Semen with a motility of between 2 and 5 were frozen in: Basic Medium (BM) +20% egg yolk (e.y), BM+6% LDL, and BM+6% LDL+60, 70, 80, 90, or 100 mmol of Glut. The spermatic and prostatic fractions were mixed and diluted in

the different freezing media at +37°C to obtain a final concentration of 100x10<sup>6</sup> spz/ml.

Exp n°2: Same way as for Exp n°1, the ejaculates were frozen in: BM+20% e.y, BM+6% LDL, and BM+6% LDL+10, 20, 30, 40, or 50 mmol of Glut.

Exp n°3: 10 ejaculates were collected from 5 dogs, and frozen in: BM+20% e.y, 6% LDL (e.y extract), and 6 % LDL+20 mmol Glut.

The semen was cooled according the classical technique.

The straws were thawed in a water bath at +37°C for 30 seconds. The semen was assessed 10 mn after thawing with a HAMILTON THORN CERROS 12.

Exp 3, spermatozoa integrity was analysed using Acridine orange (A.O), HOS, PSA-FITC, Spermac® tests.

Exp n°1: the motility results were > in the 6% LDL media (43.13%); (p<5%).

Exp n°2: the 6% LDL+20 mmol Glut gave the best results (62.1%); (p<5%, except in 10, 30 mmol Glut).

Exp n°3: All of the tests except that of A.O were superior in the 6% LDL+20 mmol Glut.

A media containing 20 mmol of glutamine combined with 6% LDL provides superior preservation of spermatozoa during the freeze-thaw process and a marked improvement in the characteristics of spermatozoa trajectories in the dog.

#### **INTEREST OF THE ASSOCIATION OF GLUTAMINE WITH LOW DENSITY LIPOPROTEIN (LDL) IN COOLING CANINE SPERM**

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Introduction: Refrigeration of canine sperm does not require specific material so is easier to use for practitioners. For a long time advantages of egg yolk (e.y) have been known in freezing semen. LDL (Low Density Lipoprotein) extracted from (e.y) play an important role in spermatozoa protection during sperm freezing. Studies have demonstrated cryoprotective action of some amino acids and especially glutamine (Glut) in conservation of stallion sperm at a concentration of 30mmol. This study aims to explore the interest of the association of LDL and Glut in cooling canine semen.

M&M: 20 ejaculates were collected from 7 dogs (Beagle, Golden retriever, Doberman) aged from 3 to 6 years. Samples were diluted in 4 media: TRIS medium (BM)+20% (e.y.), BM+6%LDL, BM+6%LDL+20mmol Glut and INRA96. Spermatic and prostatic fractions were mixed and diluted in each medium to obtain a final concentration of 100.106 spz/ml. Samples were kept at 4°C. Each day 50 fYI were removed, warmed up at 37°C during 10 mn before being assessed with a HAMILTON THORN CERROS 12. Analyses were realized during 6 - 10 days depending on the dog.4 parameters: mobility, VAP, VSL, VCL were analysed.

% of mobile spermatozoa is higher in LDL and LDL+Glut media than in INRA 96 (p<5%), however with e.y. there is a significant difference at days0, 4 and 5 (p<5%).

Discussion: Studied parameters show interests of LDL associated to Glut in cooling canine sperm which could be preserved at least 7 days after taking, while preserving the 30% mobile spz required for artificial insemination. This allows avoiding time of transport problems, time that often exceeds 2 or 3 days. Mobility percentages are always clearly superior for the LDL+Glut association.

This study demonstrates that 6%LDL+20mmol Glut permits to obtain 37.75% mobile spz 7 days after taking, percentage considered as being good enough for an artificial insemination on bitch.

#### VITRIFICATION VERSUS SLOW FREEZING: RESULTS OF A RANDOMIZED CONTROLLED STUDY OF HUMAN EMBRYO CRYOPRESERVATION

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The objective of this study was to compare the effectiveness of cryopreservation of sibling embryos after IVF utilizing either slow freezing (propanediol) or vitrification. Six or more supernumerary sibling day 2 or day 3 embryos were randomized into two groups frozen either by slow freezing or by vitrification. Decision of which group of embryos to thaw was done using a computer-generated random number sequence. There were 39 thawed cycles with the slow-freezing cryopreservation embryos and 43 cycles with the vitrified embryos. After slow-freezing the thawed intact embryos rate was 43% (59/136) and the survival rate was 84% (114/136), following vitrification the corresponding rates were 72% (95/132) and 93% (123/132). The intact and survival rates were significantly higher after vitrification as compared with the slow freezing method ( $p < 0.05$ ). In average, three embryos were transferred per patient. Clinical pregnancies were 9/39 (23%) for the slow-freezing group and 13/43 (30%) for the vitrified group (NS). Implantation rates were 9/111 (8%) and 18/120 (15%), respectively (NS). This study demonstrates a significant increase in the intact embryos and an overall significant higher survival rate with vitrification as opposed to the standard slow freezing. Yet, the implantation and pregnancy rates were comparable in both methods.

#### CLINICAL OUTCOME OF EARLY CLEAVAGE STAGE EMBRYO CRYOPRESERVATION AFTER SLOW FREEZING TECHNIQUE: OBSTETRIC AND PERINATAL DATA

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**Introduction:** Follow up of ART children has an unquestionable importance. Fewer studies about frozen-thawed embryo replacement (FER) pregnancies have been published in comparison with fresh IVF pregnancies.

**Materials and Methods:** In a retrospective study we analysed 1497 FER pregnancies (1998-2007): 1007 from the IVF programme and 490 from egg or embryo donation. Pregnancy and implantation rates after FER were 30.7% and 18.5% respectively with a mean number of 2.02 embryos per transfer.

**Results:** From the 1497 pregnancies, 1197 (79.9%) were singleton, 408 (18.3%) twins and 26 triplets (1.7%). The rates of spontaneous abortion was 408 (27.2%), 14 (0.9%) ectopic pregnancies, 13 (0.9%) therapeutic abortions, 1052 (70.3%) deliveries and 10 (0.7%) lost to follow. Selective reduction was performed in 7 (0.4%) cases. A total of 18 malformations and 6 chromosome abnormalities were reported. The female/male ratio was 1.05. No differences were observed between IVF and donor programme in any of the variables except in the Caesarean rate (52% IVF vs 71.5% donor). Significant differences were observed between singleton and multiples in neonatal mortality (14.7% vs 22.6%), preterm births (6.8% vs 45.7%) mean birth weight (3302.3 +0.62 g. vs 2435.0 +0.48g) and mean birth length (50.1 +2.46 cm vs 47.3 +3.34).

**Conclusions:** Our results do not show differences in obstetrical and neonatal data between FER pregnancies from IVF with own oocytes versus egg or embryo donation. Significant differences have been observed comparing singletons and multiple pregnancies. More follow up studies of FER pregnancies are needed to confirm the safety of the procedure.

#### FREEZABILITY DIFFERENCES AMONG EJACULATES OF THREE DIFFERENT PIG BREEDS

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The objective of this study was to compare four main sperm quality parameters among ejaculates from three different pig breeds after their cryopreservation. For this purpose, 6 Duroc ejaculates, 9 Landrace ejaculates and 8 Large White ejaculates were collected and verified to accomplish with minimal quality values before freezing. The freezability of ejaculates was quantified through analysis of the progressive motility, the viability and the osmotic tolerance of sperm at 240 min post-thawing. Volume of the sperm rich fraction was also compared. The sperm motility was captured with a computerized system of image analysis (SCA 2002, Microptic SL, Barcelona, Spain) whereas the viability was assessed with a commercial kit (Molecular probes, Eugene, OR, USA) and the osmotic tolerance was calculated as the percentage of non-reacted acrosomes in hypotonic medium. Differences among ejaculates for each parameter were tested using one-way ANOVA and Bonferroni's post-hoc tests at a significance level of  $P \leq 0.05$ . The sperm progressive motility was lower in the Large-White breed with respect to the other two breeds ( $P = 0.01$ ), and its sperm viability was also lower compared to the Duroc breed ( $P = 0.01$ ). This study shows a variation in the ejaculate freezability among breeds, for what the identification of the ejaculate traits responsible for such differences will be vital to improve the managing of boar sperm cryopreservation.

#### COMPARATIVE STUDIES OF DIFFERENT CRYOPRESERVATION METHODS FOR CHONDROCYTES AND MYOCYTES

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Proper cell cryopreservation from endangered domestic species is very important as these cells could be used to reintroduce lost genes back into the breeding pool by somatic cell nuclear transfer. In this study we have investigated the effect of different cryopreservation methods and cooling rates on primary bovine chondrocyte/myocyte viability and proliferative activity. Cells were cooled in 10% DMSO freezing media at 1°C/min, 2°C/min and 0.5°C/min rate or vitrified in a solution composed of 40% EG, 18% Ficoll and 0.3 M sucrose. Viability ratios of thawed cells were evaluated by trypan blue staining and post-thawing proliferative activity was measured by MTT assay. Results showed that slow freezing at 1°C/min cooling rate was the most appropriate method for the cryopreservation of myocytes ( $p < 0.05$ ). The viability ratios of cryopreserved chondrocytes by slow freezing at 1°C/min and 2°C/min cooling rate were higher than those cryopreserved by slow freezing at 0.5°C/min or vitrification. Cell viability decreased when 5% DMSO concentration was used at 1°C/min cooling rate. Post-thawing viability ratios of chondrocytes and myocytes were 78% and 74% at 1°C/min; 80% and 51% at 2°C/min; 61% and 51% at 0.5°C/min; 61% and 49% at vitrification. Post-thawing proliferative activity of vitrified chondrocytes and myocytes were lower than slow freezing groups. Results revealed that viability at different freezing rates and cryoprotectant concentrations changed according to the cell type. Cryopreservation protocols should be optimized for specific cell types due to different cryosensitivities.

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#### **INFLUENCE OF TEMPERATURE OF GLYCEROL ADDITION ON THE CRYOPRESERVATION OF IBERIAN IBEX (CAPRA PYRENAICA) SPERM**

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The Iberian ibex (*Capra pyrenaica*) is a wild mountain caprine originating exclusively in the Iberian Peninsula. Recently, habitat fragmentation and several outbreaks of sarcoptic mange have reduced the size of many of its populations. Thus, germplasm banks represent a valuable tool for the conservation of threatened wild species. Ibex spermatozoa have been successfully frozen using glycerolated media. However, no information is available regarding the most effective method of glycerol addition in this species. The aim of the current work was to evaluate the effect of temperature of glycerol addition on the response to freezing-thawing of spermatozoa collected by electroejaculation from six adult ibexes maintained in captivity. Each ejaculate was divided into two aliquots and frozen in a Tris-egg yolk-based medium with 5% glycerol. One fraction was directly diluted in one step at room temperature (23°C) with the freezing medium containing glycerol. The other fraction was extended in two steps, first by dilution at room temperature with identical extender without glycerol and second by adding the glycerol after cooling at 5°C. The method of glycerol addition did not affect any sperm variable after thawing. Heterospecific artificial insemination involving domestic goats was performed, for the first time, to evaluate the *in vivo* fertilizing ability of frozen-thawed ejaculated sperm from Iberian ibex. No difference was observed between the fertilization rates of spermatozoa diluted in one or two steps (18.2% vs 20.0%). These results show that temperature of glycerol addition had no influence on the cryopreservation of ejaculated ibex spermatozoa.

#### **INFLUENCE OF EGG YOLK ADDITION ON CRYOPRESERVING FOWL SPERM**

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Early reports indicated that the addition of chicken egg yolk to semen diluents decreased sperm respiration rates and fertilising potential in fowl spermatozoa. The reasons for these decreases remain unclarified but the replacement of chicken egg yolk by turkey egg yolk resulted in the disappearance of the deleterious effect. It can be hypothesised that avian intra-species interactions between the egg yolk and sperm occur in a given species. The aim of the present study was to assess the use of quail egg yolk as a novel additive for cryopreservation of fowl spermatozoa, and to compare its efficacy with chicken egg yolk based diluents and with diluents without egg yolk. Heterospermic semen samples from fourteen native Mediterranean breeds of chicken were divided into three aliquots and frozen in a PVP (polyvinylpyrrolidone)-DMA (dimethylacetamide)-based medium containing 15% chicken egg yolk, 15% quail egg yolk or without egg yolk. Cooled samples (5°C) without egg yolk showed lower viability ( $P < 0.001$ ) than those diluted with extenders containing chicken egg yolk or quail egg yolk. The thawed spermatozoa preserved with PVP-DMA-without egg yolk extender exhibited lower motility ( $P < 0.001$ ), and viability ( $P < 0.01$ ) than spermatozoa diluted with both PVP-DMA-chicken egg yolk and PVP-DMA-quail egg yolk extender. These results show that addition of egg yolk protect fowl sperm cells against cold shock and during freezing and thawing. Quail egg yolk offers no advantages over chicken egg yolk in the cryopreservation of fowl spermatozoa.

#### **MULTIFACTORIAL ANALYSIS OF PREANTRAL FOLLICLES DURING OVARIAN CORTEX CRYOPRESERVATION IN THE BITCH**

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Numerous studies have been carried on parameters affecting cryopreservation process, but few of them tried to investigate simultaneously the effects of these parameters and possible interactions existing between them. The aim of this study was to analyze combined influence of 4 different freezing parameters on the viability of preantral follicles during the cryopreservation of the ovarian cortex in the bitch. To this end, 16 female beagles (7-8 month) underwent elective ovariectomies. After dissection, ovarian cortical pieces were submitted to different cryopreservation processes according to a fractional experimental design. The simultaneous effects of the dimethylsulfoxide (DMSO 1,5M) vs propylene glycol (PROH 1,5M), sucrose vs trehalose, the post-seeding freezing rate (0,3°C/min vs 2°C/min) and the number of equilibration steps (1 step vs 3 steps) on follicles viability rate were evaluated. The experimental design revealed that sugar has no influence on viability rate. Nevertheless, cryoprotectants, freezing rate and equilibration steps highly influence the viability rate of preantral follicles ( $p < 0.0001$ ) with advantage for DMSO, at a freezing rate of 0.3°C/min and with a single equilibration bath. This combination allowed follicle viability rate to reach 85%. No interactions were revealed between these 4 factors. These results are consistent with the findings of our earlier study supporting DMSO as a good cryoprotectant for canine ovarian tissue. Furthermore, post thawing follicular morphology were assessed during this study but showed too much variability to be explored, indicating that viability assessment is currently a strong and safe indicator for freezing damages.

#### **CRYOPRESERVATION OF PREANTRAL FOLLICLES IN OVARIAN CORTICAL TISSUE FROM BITCHES (CANIS FAMILIARIS) USING DIFFERENT CRYOPROTECTIVE AGENTS**

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Cryopreservation of ovarian tissue is a tool for preservation of genetic animal resources by the female pathway. Nevertheless few studies have been carried on canine tissue. The objective of this preliminary study was to evaluate the morphological damages and the viability rates induced by the cryopreservation process on the preantral follicles in the bitch. Ovarian cortical pieces were harvested from bitches during routine ovariectomies (6-18 month,  $n=12$ ). Two permeating agents (dimethylsulfoxide 2M, DMSO or propylene glycol 2M, PROH) supplemented with or without 0.2M sucrose were used for equilibration; then, the samples underwent slow freezing process. After thawing, no significant differences were observed between control (70.2%) and DMSO groups (67.2% with sucrose, 58.2% without sucrose) when considering the number of normal follicles. In contrast, there was a decrease of normal follicle rate in the 2M PROH & sucrose group (41%) compared to control and 2M DMSO & sucrose group ( $p < 0.05$ ). The viability rate for PROH groups (23.2% without sucrose, 31.2% with sucrose) were significantly lower ( $p < 0.0001$ ) than control (91.3%) and DMSO groups (77.5% without sucrose, 82.3% with sucrose). These results demonstrate that canine ovarian cortex can be successfully cryopreserved in presence of DMSO, supplemented or not with sucrose, with a morphology and viability preservation ratio of almost 90%. Moreover, even if the morphological evaluation of the follicles corroborates the viability test results, it should always be completed by a viability assessment or some other metabolic analysis methods to refine the observed results.

## HUMAN BLASTOCYST VITRIFICATION AND WARMING: 3 YEARS EXPERIENCE USING THE CRYOTIP

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**Introduction:** Multiple pregnancies are the greatest complication of IVF treatment, and for many patients transfer of a single embryo is now recommended. However, this practice risks an overall lower pregnancy rate, when compared to cycles with multiple embryos transferred. Vitrification has the potential to allow for good recovery of preserved embryos, giving patients more confidence to transfer fewer embryos.

**Materials and Methods:** Blastocysts remaining after a fresh transfer were vitrified on day 5 and 6 post retrieval using a commercially available closed system (Vit Kit with CryoTip; Irvine Scientific, Santa Ana, CA, USA). Blastocysts at all stages (early, expanding, expanded and hatching) were vitrified and stored individually in CryoTips submerged in liquid nitrogen. Warming was on the equivalent of Day 4 in either a natural or controlled cycle with immediate transfer.

**Results:** In 467 warming cycles, 998 blastocysts were warmed on the morning of transfer. 949 embryos were recovered (95%) and from these 882 survived (94%). Overall pregnancy and implantation rates were 44% (204/467) and 29% (260/873).

| Patient age <35 donor recipients | 35-37 | 38-40 | >40 | Oocyte |
|----------------------------------|-------|-------|-----|--------|
| Cycles                           | 138   | 63    | 17  | 177    |
| Mean no. transferred             | 1.8   | 1.8   | 2.0 | 2.3    |
| Implantation rate (%)            | 35    | 24    | 24  | 25     |
| Pregnancy rate (%)               | 53    | 39    | 40  | 41     |

**Conclusions:** Confidence in embryo survival rates after vitrification allows for more aggressive use of single embryo transfer. The results of this study show that the VitKit with CryoTip is an excellent vitrification system, with the additional benefit of being a closed device.

## THE EXPRESSION OF PLURIPOTENCY AND RENOVATION REGULATORY GENES BEFORE AND AFTER CRYOPRESERVATION

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The cryopreservation of mesenchymal cells from amniotic fluid has a clinic importance due to the fact that cells collected in prenatal can be storage with the possibility of being used in immediate or even late postpartum treatments. The Oct-4, Sox-2 and Nanog genes are frequently described as the ones responsible for the regulation of auto-renovation capacity and pluripotency of embryonic stem cells, and most recent of mesenchymal stem cells. The goal of the present study is to verify the interference of cryopreservation during 5 months on the expression of those genes in mesenchymal stem cells cultivated from human amniotic fluid. Samples from nine pregnant women were included in this study. They were cultivated, characterized by immunophenotypic profile and differentiation capacity, and submitted to cryopreservation for 150 days. To verify the cryopreservation interference over embryonic genes expression, we compared the expression of those genes 150 days before and after cryopreservation. The expression of Sox-2 and Nanog genes and Oct-4 gene were evaluated qualitatively and quantitatively, respectively. The observed results from Sox-2 and Nanog did not show qualitative alteration before or after cryopreservation. Expression level variation was observed in Real Time PCR experiments;

however, this variation did not follow a pattern, being lower in 5 patients and higher in 4. In conclusion, the process of cryopreservation did not alter the expression of those genes, therefore did not influence in regulatory processes of OCT-4, SOX-2 and Nanog.

## SURVIVAL OF BOVINE OOCYTES TO VITRIFICATION AFTER HYDROSTATIC SUBLETHAL PRESSURE TREATMENT

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This work analyzes the use of the high hydrostatic pressure (HHP) method to improve survival rates to vitrification of bovine oocytes. Bovine cumulus oocyte complexes (COCs) were in vitro matured for 22 h and divided into two groups: 1) untreated controls; and 2) HHP treated (200 bar, for 60 min at 27°C), followed by a recovery period of 60 min in IVM medium. The COCs were vitrified (Diez et al., *Theriogenology* 2005; 64:317-333) in fiberplugs. Warmed COCs were in vitro fertilized, and subsequently cultured up to Day 8 in B2+10% FCS with Vero cells. Two groups of fresh COCs (untreated, and HHP treated) served as controls. Development rates were recorded on days 3, and 6 to 8, analyzed by ANOVA and Duncan test, and data presented as LSM±SE. Vitrification significantly reduced development. In our hands, HHP treatment of oocytes did not affect Day-8 blastocyst rates ( $p>0.05$ ) both as fresh ( $31.1\pm 4.8$  vs  $30.5\pm 6.1$ , for controls and HHP, respectively) and vitrified/warmed ( $7.1\pm 3.9$  vs  $5.5\pm 4.3$ , for controls and HHP respectively). Modifications in the HHP treatment protocol (duration, temperature or pressure) must be made in an attempt to improve oocyte survival after cryopreservation. INIA RZ-2008-00014; RTA2008-0082. M. Munoz, B. Trigal and D. Martin are sponsored by RYC08-03454, Cajastur and PTA2007-0268-I, respectively

## DEVELOPMENT OF VITRIFIED/WARMED BOVINE OOCYTES AFTER PARTHENOGENETIC ACTIVATION OR IN VITRO FERTILIZATION

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Cryopreservation of bovine oocytes results in compromised survival rates. Exposure of oocytes to conventional permeating cryoprotectants increases calcium levels, which may trigger oocyte activation leading to zona hardening (ZH) (Gardner et al, *Theriogenology* 2007 67:64-72). This work analyzes the use of parthenogenetic activation (which could avoid putative fertilization problems due to ZH) as a way to improve developmental ability of the oocyte after vitrification in fiberplugs. In vitro matured, vitrified/warmed bovine cumulus oocyte complexes were parthenogenetically activated (Gomez et al., 2009; 137:285-295) or in vitro fertilized (IVF), and subsequently in vitro cultured until Day 8 in B2+10% FCS with Vero cells. Two groups of fresh COCs (activated and IVF) served as controls. Development rates were recorded on days 3, and 6 to 8, analyzed by ANOVA and Duncan test, and data presented as LSM±SE. Parthenogenetic activation did not affect fresh oocyte developmental ability (Day 8 blastocyst rates:  $31.2\pm 4.0$  vs  $35.0\pm 7.5$ , for IVF and activated fresh oocytes respectively;  $p>0.05$ ) neither after vitrification/warming (Day 8 blastocyst rates:  $7.1\pm 3.3$  vs  $13.2\pm 3.3$ , for IVF and activated fresh oocytes respectively;  $p>0.05$ ). Vitrification significantly reduced ( $p<0.05$ ) developmental rates, therefore vitrification protocols should be revised in an attempt to improve oocyte cryopreservation success.

INIA RZ-2008-00014; RTA2008-0082. M. Munoz, B. Trigal and D. Martin are sponsored by RYC08-03454, Cajastur and PTA2007-0268-I, respectively.

**COMPARISON OF POST-THAW/WARMING SURVIVAL RATES, REEXPANSION, AND PREGNANCY OUTCOMES BETWEEN VITRIFICATION AND SLOW-FREEZING METHODS FOR HUMAN BLASTOCYST CRYOPRESERVATION**

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Objective: To compare the effectiveness of vitrification and slow-freezing for human blastocyst cryopreservation.

Design: A retrospective analysis of frozen blastocyst transfer (FBT) cycles between Sep. 2005 and Dec. 2009.

Materials and Methods: Blastocysts were developed in Quinn's advantage culture media during IVF cycles; then cryopreserved with slow freezing before May 2007 and with vitrification (cryotop) technique after May 2007. Cryopreserved embryos were thawed/warmed and cultured for 2 to 6 hours before transfer. Post-thawing/warming survival rates and reexpansion during culture were analyzed. Pregnancy outcomes were also compared between two cryopreservation methods.

Results: In total, 201 consecutive FBT cycles were analyzed. One hundred and twenty-nine (129) used blastocysts cryopreserved with slow-freezing and 72 with vitrification. Total numbers of thawed/warmed embryos were 327 and 160 with slow-freezing and vitrification methods, respectively. The survival rate for vitrification (98.8%) was significantly higher than for slow-freezing (93.6%,  $p=0.022$ ). There was also a higher percentage of reexpanded blastocysts (reaching  $\geq 80\%$  of their original size) for vitrified embryos (85.6%) than for slow-frozen embryos (67.0%,  $p=0.0001$ ) after 2 to 6 hours of culture post-thawing/warming. However there was no difference in ongoing pregnancy rates between the two groups (vitrification 52.8% vs slow-freezing 54.3%,  $p=0.95$ ). Implantation rate was slightly higher for vitrified than slow-frozen embryos (38.3% vs 33.5%), but the difference did not reach statistical significance ( $p=0.37$ ).

Conclusion: Vitrification is at least as effective as slow-freezing for cryopreserving human blastocysts. Blastocysts cryopreserved with vitrification had better survival and reexpansion rates; but implantation potential for transferred embryos was similar with both methods.

**IMPROVED EMBRYOID BODY CRYOPRESERVATION AND CARDIOMYOCYTE DIFFERENTIATION FOLLOWING HIGH HYDROSTATIC PRESSURE TREATMENT**

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Embryonic stem cells (ESC) are powerful tools for research on regenerative medicine. In most protocols in order to induce differentiation of ESCs in vitro, first Embryoid Bodies (EBs) are produced. Therefore cryopreservation of EBs as intermediate forms prior to production of fully differentiated cells, would be highly desired. High hydrostatic pressure (HHP) applied as a sublethal stress treatment may induce stress tolerance of the cells. Here we report the first results of EB cryopreservation combined with HHP.

Mouse ESCs with 129/SvJ origin were used to prepare EBs using hanging drop technique. 96 EBs were assigned to four experimental groups (HHP treated/not treated, with or without vitrification) and 4 repetitions were made. Treatment was performed at 600 bar for 30 min at 24°C in a computer controlled pressurizing device (Cryo-Innovation Inc). After vitrification/warming by solid surface vitrification (SSV; Dinnyes et al., 2000), the EBs were placed onto gelatin coated dishes. The survival rate and capability of differentiation into cardiomyocytes were determined.

Our studies showed that the pressure treatment did not affect the survival and the differentiation rate in the non-vitrified groups. The HHP treatment had no effect on the post-warming survival rate of the EBs, however, highly improved the efficiency of in vitro differentiation towards

cardiac lineages (74,6% vs. 36,9%, respectively).

In conclusion SSV combined with HHP is a highly suitable method to cryopreserve EBs and to improve the efficacy of cardiac differentiation from cryopreserved samples.

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**TNF ALPHA -308 A/G POLYMORPHISM AND IVF OUTCOME**

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Objective: The aim of this study was to examine the genotype distribution of the -308 TNFalpha gene polymorphism; in 401 women with couple infertility requiring ICSI procedure for various aetiologies.

Material and methods: We used the ASPCR to identify the polymorphism, and the same time the patient hormonal status was studied through the evaluation of plasma levels of FSH and the E2, and the ratio E2/FSH.

Results: Our study observes. The TNF-308.2 allele is associated to an increase implantation rate with an increased the multiple pregnancy rate especially in the case of 3 embryos transfers.

Conclusion: Our present observation raises the possibility of an oval potential use of the anti TNFalpha agents in the beginning of the hyper stimulation and the necessity to adapt the transfer protocol for women with TNF-308.allele

**OBTAIN HESC LINE UNDER ANIMAL-FREE CONDITION FROM BLASTOMERS AFTER PGD**

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Up to this date, the existing methods has been based on cultivating on MEF. It leads to contamination cells by animals' agents, increases risk of infection and makes standartization of differentiation protocols impossible. All of these limit using such ESCs in clinic. To overcome this difficulty we tried to obtain hESC line from remained after PGD blastomers under animal-free condition with the help of mTeSR medium. Every blastomer was taken and put into free zona pelucida after blastocysts hatching. These constructions were incubated in standard medium for embryology (Cook) or medium for ESCs (mTeSR-1, StemCellTech) with added inhibitor of Rock kinase Y27632 for 5-6 days. We obtained 34 blastomers and they were transferred into zona pelucida. Media were changed daily. In six days we received only one blastocyst, 4 morulas only in mTeSR medium. Also we obtained 4 two- and three-cellular constructions with the help of mTeSR. However, these constructions degraded the next day except blastocyst and morulas. All blastomers in embryology media did not develop into blastocysts and morulas. They could turn into two-cell construction. To obtain ESCs we used osmotic shock to demolish trophoblast and put ICM or morulas on Matrigel in mTeSR. Only ICM developed in ESC colony in two weeks. ESCs formed monolyer and passaged with the help of disperse solution. We tested presense of oct-4, nanog, SSEA, TRA-1-60 in ESCs. After the third passage, ESCs were cryopreserved under standard condition – 10% DMSO, 10% SR and medium.

## EFFECT OF ADDITION OF REDUCED GLUTATHIONE ON LIPID PEROXIDATION IN THAWED BOAR SEMEN

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All living cells under aerobic conditions produce reactive oxygen species (ROS). These substances may induce cellular damage. Polyunsaturated fatty acids are especially sensitive; ROS induced damage leading to lipid peroxidation that seriously compromises sperm structure and function. The aim of this study was to test the effect of an antioxidant to reduce the damage caused by ROS during conservation of pig sperm. The ejaculates of 10 boars were frozen in two different groups, with 5 Mm glutathione and control. Motility was assessed using a CASA. Analysis of lipid peroxidation was performed using the Thiobarbituric Acid Assay (TBARS). Sperm vitality was tested using a staining by propidium iodide/carboxyfluorescein and acrosomal status was evaluated with phase contrast microscopy, all tests were made the same day. The Results were expressed as a means  $\pm$  SD. Total motility in controls was  $48.77 \pm 21.57\%$ , glutathione supplemented samples was  $51.07 \pm 2.93$ . Intact acrosomes in control were  $85.12 \pm 4.96\%$  vs  $91.10 \pm 2.96\%$  ( $p < 0.001$ ). Sperm vitality was  $68.66 \pm 8.25\%$  in control vs  $77.46 \pm 2.99\%$  ( $p < 0.001$ ) in supplemented samples. Lipid peroxidation was in controls  $0.48 \pm 0.08$  vs  $0.35 \pm 0.07$  mmoles TBARS/ $10^8$  sperm ( $p < 0.001$ ) in glutathione group. The use of glutathione improve the semen quality after thawed.

## VITRIFICATION IS AN EFFECTIVE CRYOPRESERVATION TOOL FOR HUMAN CLEAVAGE-STAGE EMBRYOS

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Objective: Human embryo cryopreservation by vitrification has received increased attention in recent years. Although the technique has initially been preferred for oocyte and blastocyst-stage embryo freezing applications, recent data suggest that it may also be effectively applied in all stages of human embryo development. Here we report our preliminary data on day 3 embryo vitrification/warming cycles from a private hospital-based IVF Center in Turkey.

Material/Methods: During the study period, supernumerary good quality embryos or embryos of cycles in which total embryo freezing were required were vitrified and warmed by using ethylene glycol/DMSO-based commercial vitrification and warming kits (Irvine scientific). Sequential embryo culture system in microdroplets (G1.3/G2.3, Vitrolife) was used for embryo culture. Embryo warming was performed one day before embryo transfer and developing good quality embryos were selected for uterine replacement.

Results: Between January 2008 and November 2009, a mean number of  $6.3 \pm 3.1$  supernumerary embryos were selected for vitrification in 212 IVF cycles. In 62 warming cycles, a mean no of 4.3 embryos were warmed with 85.1% survival and 95.2% further cleavage rates. Embryo transfer was performed in all warming cycles with a mean of 2.8 embryos transferred, resulting in 40.3% pregnancy and 15.2% implantation rates respectively.

Conclusion: Based on embryo survival, development and clinical outcome, our preliminary results can show that vitrification is an efficient method of cryopreservation of human day 3 embryos. More studies with larger sample sizes are needed to independently assure the safety and efficiency of different vitrification systems on early embryo development.

## VITRIFICATION OF BOVINE IN VITRO-PRODUCED EMBRYOS

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In this trial we aimed to compare the subsequent pregnancy rate of vitrified and fresh in vitro-produced embryos. Embryos were produced by 9 Friesian cows through transvaginal recovery and in vitro production (IVP) over a 10-week period. The embryos produced during the first 6 weeks were all vitrified and warmed during the last 4-week period concurrent with the transfer of fresh embryos produced from the same donor cows at that time. Vitrification and warming were performed using a technique previously reported (Peachey B et al. 2005 *Reprod. Fertil. Dev.* 17, 199 abst) using the CVM method (Lindemans W et al. 2004 *Reprod. Fertil. Dev.* 16, 174 abst). All embryos were transferred by the same experienced technician into randomly assigned synchronized recipients of the same herd. All recipients were scanned for pregnancy 40 days after transfer. Data (Table 1) were compared by Fisher exact test and showed no significant difference ( $P = 0.761$ ) between pregnancy rates of vitrified-warmed embryos and fresh embryos. This result demonstrates that vitrification is a valuable technique in cryopreservation of IVP embryos.

Table 1. Pregnancy rates of fresh vs. vitrified/warmed IVP embryos

|                        | Fresh    | Vitrified |
|------------------------|----------|-----------|
| Embryos at Day 7, n    | 112      | 130       |
| Embryos transferred, n | 112      | 117       |
| Pregnant, n (%)        | 59 (53%) | 65 (56%)  |

## THE KINETICS OF CHILLING INJURY IN SPERM IS MODERATED BY CHOLESTEROL AND EGG YOLK

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Chilling injury in ram sperm was measured by motility changes after exposure to  $0^{\circ}\text{C}$  for different durations. Using a pneumatic piston and time controller we cooled 2ul of semen in small glass capillary sealed in the bottom. A thermocouple was inserted inside the capillary and was inserted into liquid nitrogen for 0.5 to 1 seconds in  $0^{\circ}\text{C}$  Celsius or into ice water for 180 seconds exposure. After warming the sperm was evaluated in a Meckler chamber under a light microscope.

We found that ram semen are very sensitive to chilling injury and 0.5, 1 or 180 seconds exposure to  $0^{\circ}\text{C}$  decreased motility to  $53.2 \pm 12.5$ ,  $11.3 \pm 8$  and  $2.6 \pm 2.5\%$  respectively. When reducing membrane cholesterol by using Beta-cyclodextrin, the exposure to  $0^{\circ}\text{C}$  was lethal to ram semen. Addition of cholesterol-loaded cyclodextrin (CLC) or egg yolk delayed the chilling injury of ram sperm to 180 seconds (PCM= post chilling motility of  $30 \pm 3.8\%$  and  $31 \pm 10\%$  respectively), while the addition of both egg yolk and CLC had a synergistic effect (PCM= $47 \pm 1\%$ ).

Human sperm is less susceptible to chilling, with some individual differences. A reduced in sperm motility was found when sperm was exposed to  $0^{\circ}\text{C}$  for more than 1 minute (PCM= 50% after 10 seconds and 15% after 1 minute). CLC and egg yolk preserved the motility of human sperm similarly to ram semen.

Lipid phase transition was measured using microscopic FTIR showed that ram semen has a central transition- $T_m$  at  $17^{\circ}\text{C}$  while human has  $T_m$  at  $9^{\circ}\text{C}$ . Adding CLC to ram sperm reduces the  $T_m$  by  $10^{\circ}\text{C}$ .

### INITIAL RESULTS OF EMBRYO CRYOPRESERVATION AFTER THE INTRODUCTION OF VITRIFICATION IN A CLINICAL IVF PROGRAM

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**Objective:** To assess the initial results of the vitrification technique (VT) as an alternative to slow freezing (SF) for cryopreservation of embryos produced in a clinical IVF program.

**Material and Methods:** IVF cycles with cryopreservation (n=199) were pseudorandomized at a ratio 1:3 (SF:VT) according to day of the week, on a prospective, open label design. Only good quality embryos were cryopreserved. For SF (n=66 cycles) we used freeze kit media (Vitrolife), CBS straws, and a programmable freezer (Planer 1.7). Vitrification (n=133 cycles) was done using cooling media (Medicult) on Cryoleaf straws (McGill). Thawing and warming were done, respectively, with media from Vitrolife and Medicult, the day before transfer to assess the survival and growth of the embryos.

**Results:** We analyzed the results of 72 cycles of thawing corresponding to SF (n=233 embryos) and 48 warming cycles of VT embryos (n=118). The survival rate was 76% with SF and 85% with VT. The percentage of growing embryos was 60% (SF) and 67% (VT), and the pregnancy rates per cycle and per transfer were 33% and 35% (SF) compared with 26% and 30% (VT) respectively. Implantation rates were 35% (SF) and 30% (VT). Finally, 23% of pregnancies obtained from SF embryos ended in abortion, while none of the pregnancies from VT embryos aborted (p<0.01).

**Conclusions:** The technique used during the initial implementation of vitrification in our laboratory shows equivalent results as compared with the classical slow freezing method. Vitrified embryos, once implanted, showed better developmental profile than slow frozen embryos.

### INITIAL RESULTS OF EMBRYO CRYOPRESERVATION AFTER THE INTRODUCTION OF VITRIFICATION IN A CLINICAL IVF PROGRAM

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**Objective:** To assess the initial results of the vitrification technique (VT) as an alternative to slow freezing (SF) for cryopreservation of embryos produced in a clinical IVF program.

**Material and Methods:** IVF cycles with cryopreservation were pseudo-randomized at a ratio 1:1 (SF:VT) according to day of the week, on a prospective, open design. Only good quality embryos were cryopreserved at cleavage stage (D2 or D3). For SF (n=212 embryos) we used Freeze-kit 1™ (Vitrolife), CBS straws, and a programmable freezer (Planer 1.7). Vitrification (n=187 embryos) was done using Vitrification Cooling media (Medicult) and Cryoleaf straws (McGill). Thawing (30 cycles, 84 embryos) and warming (51 cycles, 126 embryos) were done, respectively, with Thaw-kit 1™ and Vitrification Warming, the day before transfer to assess the growth potential of the embryos.

**Results:** The survival rate was 82% with SF and 87% with VT. The percentage of growing embryos was 60% (SF) and 67% (VT). A mean of 1.9 and 2.0 embryos were transferred, respectively, in the group of SF and VT. The pregnancy rates per transfer were 20% (SF) compared to 28% (VT). Implantation rates were 12% (SF) and 20% (VT). All the pregnancies in the SF group were single, whereas 4/12 pregnancies in the VT group contained >1 sac. One pregnancy (16%) from SF embryos ended in abortion, while none of the pregnancies from VT embryos aborted.

**Conclusions:** The technique used during the initial implementation of vitrification in our laboratory shows equivalent results as compared with the classical slow freezing method.

### EFFECT OF CRYOPROTECTANT AND CHILLING ON OXIDATIVE STRESS IN ZEBRAFISH (DANIO RERIO) EARLY OVARIAN FOLLICLES

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Oxidative stress is associated with poor gamete and embryo quality in mammals however there are few studies on fish. Increase in reactive oxygen species (ROS) and disruption in ATP production play important roles in cell signalling and apoptosis. Catalase is the principal scavenger of ROS in the presence of hydrogen donor substances such as methanol in the cells. In present study, we investigated the effect of methanol treatment and chilling on catalase gene expression, ROS activity and ATP levels in stage I zebrafish ovarian follicles (7-140 µm). Follicles were exposed to 2M and 3M methanol for 4h at 22°C or up to 24h at 2.5°C. ROS staining involved incubation in 25 µM CH2DCFDA for 30 min at 22°C, followed by examination using fluorescent microscopy. Catalase gene expression was assessed using real time PCR. SYBR green fluorescence was used to quantify gene expression levels relative to housekeeping genes, EF1α and β-actin. ATP measurement of follicular extracts from each treatment was carried out using a ATP Bioluminescent Assay Kit.

ROS activities significantly increased following incubation for 4h at 22°C under control conditions and exposure to 2M methanol, compared to that zero control and after 4h incubation at 2.5°C (p<0.05).

A significant decrease in catalase gene expression and increase in ATP level was observed in follicles exposed to 3 M methanol for 4h at 2.5°C compared to follicles incubated at 22°C and the controls at time zero (p<0.05).

### ALTERED GENE EXPRESSION IN EMBRYO OBTAINED WITH DNA-CRYODAMAGED SPERM

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Different studies have shown that sperm cryopreservation could promote DNA damage, reporting base oxidation and strand breaks. The use of artificial reproductive techniques bypasses physiological barriers responsible for sperm selection, increasing the risk of fertilization by damaged spermatozoa, especially when sperm from subfertile males is used. Fish seem to have weaker selection mechanisms, are very prolific and have external embryo development, being convenient models for the study of the effects of DNA damage on the offspring. We cryopreserved rainbow trout semen obtained near the end of the reproductive season, when susceptibility to suffer DNA cryodamage increases. DNA fragmentation was analyzed using Comet assay, and fertilization trials were performed with fresh and cryopreserved spermatozoa. Embryonic mRNA were extracted at somites, eyed and larvae stages. The expression of eight genes from the gonadotropic axis (IGF-1, IGF-2, IGFR1a, IGFR1b, INS-1, INS-2, GH-1 and GH-2) was analyzed using Real-time PCR. Results showed that all the cryopreserved spermatozoa carried at least 10 % fragmented DNA (no spermatozoa in the 0-10% category). Fertilization rates with fresh and frozen sperm were 90.6 ± 2.5% and 61.1 ± 2.3%, respectively, and hatching rates 78.3 ± 5.4% and 14.5 ± 4.4%. Thus, frozen sperm yielded a higher rate of abortions. In these batches, surviving embryos displayed abnormal gene expression: expression of IGF-1, IGFR1a, IGFR1b, GH-1 and GH-2 was enhanced after hatching, demonstrating changes in the control of gene expression when DNA-damaged cells fertilized the oocytes. We conclude that fertilization with cryopreserved DNA-damaged spermatozoa significantly affects the offspring performance.

### ICE-FREE CRYOPRESERVATION: NEW SIMPLIFIED METHOD HAS NO INFLUENCE ON STRUCTURAL INTEGRITY OF BLOOD VESSELS

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Conventional frozen cryopreservation (CFC) via controlled rate freezing and storage above liquid nitrogen is state of the art for preserving cardiovascular tissue. The objective of this study was evaluation of a novel simplified method for cryopreservation of blood vessels: Ice-free cryopreservation (IFC) requires less sophisticated equipment and storage facilities and therefore is more cost-effective than CFC.

Porcine aortic artery segments were procured from a local slaughterhouse. IFC tissues were infiltrated with 12.6 M cryoprotectant solution containing 4.65, 4.65 and 3.31 mol/L of DMSO, formamide and 1,2-propanediol. Bags were cooled for 10min at -120°C and stored at -80°C. CFC was realized in DMEM with 10% DMSO and slow rate frozen and stored in vapour-phase nitrogen using standard protocols. Fresh tissues were kept in DMEM with antibiotics at 4°C. After warming/thawing and washing the vessels were processed for histology (H&E-staining and Movat-Pentachrome). Collagen and elastin was studied in samples without chemical fixation via two-photon laser scanning microscopy (LSM). A panel of haemocompatibility tests was also performed.

Conventional Histology and LSM showed well preserved extracellular matrix (collagen and elastin) architecture within IFC vessels. Haemocompatibility studies of platelets, thrombin/antithrombin-III-complex,  $\beta$ -thromboglobulin, inflammation marker PMN-elastase and terminal complement complex SC5b-9 demonstrated no significant differences between fresh, IFC and CFC tissues.

These results indicate that IFC can be a cost-effective storage and shipping alternative to CFC as it avoids risks of cracking and ice-induced matrix damage. The unaltered haemocompatibility results compared with fresh samples supports IFC as a cryopreservation method for vascular implants.

### FREEZING CAUSES AGING?: TELOMERE LENGTH IN STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

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Actually an important investigation field in Odontology is the use of oral stem cells to regenerate dentino-pulpar complex, in repair osseous defects or corneal epithelium, and yet repair central nervous system, even recently has been created "Tooth Banks". However if pretend that the culture cells from cryopreserved tissues can use in therapeutic applications, is necessary find methodologies to allow assuring its viability and function. The telomere length allows us to know if a cell is young or old, because in aged cells a telomere shortening occurred, and it induce replicative senescence. Then the telomere length can use as a tool to know the replicative cell age after cryopreservation. At the present work the telomere length was measured in stem cells from human exfoliated deciduous teeth by Q-FISH and TRAP, after short periods of cryopreservation 1 day, 7 days and 1 month in trehalose, glycerol and DMOS, a diminution in telomere length related to the type of cryopreservant was observed, the results indicated that the trehalose preserves the telomere length, and therefore the viability and cellular function in a percentage greater than glycerol and the DMSO. The cultures were characterized with specific antibodies STRO-1 and CD34 and analyzed by CLSMS.

### ULTRASTRUCTURAL EVALUATION OF SLOW FROZEN-THAWED HUMAN OVARIAN TISSUE

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**Objective:** The aim of this study was to observe the histological and ultrastructural characteristics of preantral follicles of frozen-thawed ovarian cortical tissue after exposure of ovarian tissue to cryopreservation in dimethyl sulfoxide, cryostorage in Nitrogen and thawing.

**Materials and Methods:**

The ovarian cortex biopsies of each ovary were obtained from 6 patients with breast cancer involved in the Fertility Preservation Program of Hospital Universitario Doctor Peset. Ovarian fragments of 1 mm<sup>2</sup> were processed and cryopreserved following slow freezing protocols. One month later, the samples were thawed using three steps.

Tissue fragments with a maximum dimension of 1 mm<sup>3</sup> were fixed. The ultra-thin sections were contrasted with uranyl acetate and lead citrate, and examined under transmission electron microscope.

**Results:** In frozen-thawed ovarian biopsies analyzed by LM-TEM, the observed follicles showed preserved cytologic architecture, they were small in diameter, with the characteristics of primordial and primary follicles. The oocyte and the follicular structures generally seemed well preserved. Flattened follicle cells surrounded the oocyte. Endoplasmic reticulum and lipid bodies were associated closely with an intact nuclear membrane. In the central oocyte cytoplasm, there was accumulation of mitochondria (Balbiani vitellin body), suggesting that it was not damage to the mitochondria after cryopreservation. Occasionally, follicular cells were destroyed with cytoplasm occupied by vacuoles, and membrane damage in mitochondria was observed.

**Conclusions:** Transmission electron microscopy confirmed the integrity of the primordial and primary follicles with our freezing / thawing protocol for cryopreservation of ovarian cortex and after exposure of ovarian tissue to cryopreservation in DMSO.

### SUCCESSFUL VITRIFICATION OF MOUSE OOCYTES BY CRYOTOP

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Unfertilized oocytes are one of the most desired germ cell stages for cryopreservation because these cryopreserved oocytes can be used for assisted reproductive technologies, including in vitro fertilization or intracytoplasmic sperm injection. However, in general, the fertility and developmental ability of cryopreserved oocytes are still low. The aim of the present study was to improve vitrification of mouse oocytes. The effects of calcium and cryoprotectants (dimethyl sulfoxide and ethylene glycol) in vitrification medium on the survival and developmental ability of vitrified oocytes were evaluated. Additionally, the role of cumulus cells on the vitrification was also examined. Oocytes were equilibrated in 15% cryoprotectants + 20% fetal calf serum (FCS) for 3 min and then 30% cryoprotectants + 0.5 M sucrose + 20% FCS for 1 min, before being plunged into liquid nitrogen on Cryotops. Most of the vitrified oocytes were morphologically normal after warming but were not fertilized and developed independently of calcium and cryoprotectants. The fertility and developmental ability of denuded oocytes after in vitro fertilization were low, even in fresh oocytes. Vitrified oocytes with cumulus cells showed significantly ( $P < 0.05$ ) higher fertility and ability to develop into blastocysts than those of vitrified oocytes. Taken together, the current results clearly demonstrate that unfertilized oocytes with cumulus cells can be successfully vitrified by Cryotop using calcium-free and ethylene glycol supplemented media.

## PROCESSING AND CRYOPRESERVATION OF 708 UNITS OF HUMAN UMBILICAL CORD BLOOD

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The study was aimed to establish a standard procedure for collection, separation, enumeration and cryopreservation of cord blood hematopoietic stem cells at the human umbilical cord blood bank. 708 samples of cord blood were collected after delivery of infant prior to expulsion of placenta. The average cord blood volume collected was 102.6 ml. All cord blood units were processed by using the Sepax automated method (Biosafe, Switzerland). The recovery rate of nucleated cells and mononuclear cells was 79.63±19.3% and 77.9±21.7% respectively, average number CD 34+ cells was 22.2 x 10<sup>5</sup> per unit, viability count of mononuclear cells was 99.31%. After finishing all tests and adding of 10% DMSO cord blood units were transferred to a controlled rate freezer (Planer, UK) that was pre-cooled to 4±186°C. The units were then cooled at 1±186°C/min to -12±186°C, cooled at 20±186°C/min to -60±186°C, followed by warming of the units at 10±186°C /min to -18±186°C, cooled at 1±186°C/min to -60±186°C and finally, 3±186°C/min to -100±186°C. Then cord blood units were stored in the liquid nitrogen for further application. Seven units of cord blood were thawed after one year of cryopreservation, the recovery rate of nucleated cells and CD34(+) cells were 95.5±3 % and 85.2±23 % respectively and the viability count on revival of mononuclear cells was 85.9%.

It is suggested that the methods reported for processing and cryopreservation of hematopoietic stem/progenitor cells in the human umbilical cord blood is effective.

## AN ANIMAL/HUMAN COMPONENT FREE IN FREEZING MEDIUM FOR RABBIT EMBRYOS

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Most freezing media contains a surfactant of animal origin, such as albumin or sera (BSA, FCS, NBCS...) which could be a potential vector for pathogens or scrapie.

A new protein substitute, Stem α945; Cryo III (www.stemalpha.com) is a well defined biochemical medium without animal components, which was developed for human medicine for the freezing of somatic stem cells (mainly hematopoietic stem cells).

The aim of our work was to replace BSA by Stem α Cryo III in the freezing medium for rabbit embryos. Overall, 374 morulae were pooled to test 6 freezing media: a control group containing PBS + 4 g/l BSA ; and 5 other groups with different concentrations of stem α945; Cryo III in PBS (20%) and pure water (20%, 40%, 60% and 80%). Embryos were slow frozen and stored at least 2 months in LN2. After thawing and 48 h in vitro culture (in MEM + 10% serum at 38,5°C and 5% CO<sub>2</sub>) respectively 98% a, 86% ab, 53% d, 54% d, 78% c and 80% bc of thawed morulae reached expanded blastocyst stage. Results were compared using chi-square analysis: percentages with different superscript are significantly different (p<0.05).

This experiment indicates that stem α945; Cryo III could be used successfully to replace protein of animal origin in freezing media, and thus improve sanitary level in the embryo transfer industry.

## OUTCOMES AFTER SLOW-FREEZING OF HUMAN BLASTOCYSTS OF DAY 5 AND DAY 6

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Introduction: Our results have shown that day 6 blastocyst have a slightly lower success rate than day 5 blastocysts. To determine if this difference is due to the quality of embryos or endometrium, we compared the results obtained from frozen embryos of different days.

Materials and methods: 360 cycles IVF/ICSI were performed in our clinic between January 2008 and December 2008. In 35 cycles with day 5 blastocysts (group A) and in 32 cycles with day 6 blastocysts (group B) all good quality supernumerary embryos were cryopreserved and then thawed for replacement in uterus. We compared survival, implantation, clinical pregnancy and ongoing pregnancy rates in both groups. Estradiol and vaginal progesterone without ovarian stimulation were used to prepare recipient's uterus. Mean number of transferred embryos was 2.2.

Results: After warming 60% (n=35) and 62.5% (n=32) of embryos survived in group A and B respectively. 33 embryos transfers were performed in group A and 32 – in group B. Implantation rate in freeze-thaw cycles was 11% in group A and 12.3% in group B. Clinical pregnancy rate per embryo transfer was similar in both groups: 21.2% (7/33) and 21.8% (7/32) in group A and B, respectively. Ongoing pregnancy rates were 15.2% and 18.7% in studied groups.

Conclusion: In our brief studies, we found that the potential of implantation is independent of the day 5 and day 6 embryos. Day 6 blastocysts do not have a negative effect on the implantation potential or the outcome of pregnancy compared to day 5 blastocysts.

## EFFECT OF THAWING TEMPERATURES ON CASA PARAMETERS AND ACROSOMAL INTEGRITY OF FROZEN-THAWED STALLION'S EPIDIDYMAL SPERMATOZOA

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Cryopreservation of male gametes is an important tool in assisted reproductive techniques; long-term storage of spermatozoa is necessary when in vitro fertilization (IVF) or artificial insemination is to be performed at a future date. Cryopreservation of epididymal spermatozoa offers a potential tool for rescuing genetic material from males of genetically elite populations. Castration, catastrophic injury, sudden death or any other event that makes semen collection or mating impossible may prematurely terminate a stallion's reproductive. Stallion epididymal spermatozoa vary widely in the loss of progressive motility, in acrosomal integrity, and in viability during freezing and thawing. The objective of this work were to: (1) the effects of thawing temperatures (37, 56, or 70 °C) on Computer Assisted Sperm Analysis (CASA) parameters and (2) on acrosome integrity of stallion post-thaw spermatozoa, (3) the effects of thawing temperature (37, 56, or 70 °C) and post-thaw incubation time (0, 1, 2, 4 or 8h) on castrated epididymis of stallion's. Post-thaw motility ranged between 58.0% (56 °C) and 64.3% (70 °C) in the various thawing times. Higher percentages of motile and rapid sperm were observed at 37 °C thawing temperature (P<0.05). As the thawing temperature was increased the number of sperm with an intact acrosome decreased. The motility, VCL (Velocity of Curvilinear Line), VAP (Velocity of Average Path) and BCF (Beat-Cross Frequency) of post-thawed sperm decreased significantly by increasing the incubation time for all thawing temperatures.

The present study showed that stallion's epididymal spermatozoa thawed at 37°C for 1min resulted in the highest motility rates, velocity of movement and higher rate of intact acrosomes.

sealed in cryobags and frozen in controlled rate freezer. Presented technique allows for routine collection and banking of hematopoietic stem cells and mesenchymal stem cells from the same donor for potential clinical applications.

#### **CRYOPRESERVATION OF CORD BLOOD HEMATOPOIETIC STEM CELLS – BULGARIAN EXPERIENCE**

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The collection, processing, phenotyping, program freezing and storage of hematopoietic stem cells derived from umbilical cord blood has been carried out at the Cryobank of the Center for Human Reproduction "Dimitrov" in Sofia since November 2006. The stem cells bank has linked by partner collaboration several research units and IVF-clinics in Sofia, which allowed for the development and implementation of the science-based biotechnology into the clinical practice at large. Until now, there have been more than 350 samples of cord blood processed and stored in the Cryobank, which operates under the strict control of the Bulgarian Executive Agency for Transplantation in accordance with the national and European legal regulations. Retrospective data analysis has shown that the obtained umbilical cord blood volume, percentage of stem cells recovered and their viability after thawing are comparable to the results reported by world's leading cryobanks of hematopoietic stem cells. The average volume of the obtained and processed cord blood has been 93.4 ml (between 42 and 166 ml) and the average percentage of collected CD34+ cells - 0.42 % (between 0.11 and 1.56%). About 81 percent of the hematopoietic stem cells have retained their viability as well as their colony-forming activity after programmed cryopreservation as demonstrated by specific assays (Annexin V/PI, CFU-E, BFU-E, CFU-GM, CFU-GEMM). Our data have provided evidence that the stored samples might be a reliable source of human pluripotent cells for future application.

#### **BANKING OF CORD BLOOD CELLS (CBC) AND UMBILICAL CORD STROMAL CELLS (UCSC) FOR POTENTIAL CLINICAL APPLICATIONS**

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The rationale of the project is to bank the CBCs and UCSC from the same donor for clinical transplantation and GVHD prevention or treatment. CB cells were collected according to the routine protocol applied by cord blood bank: CB was collected by aspiration into blood bag following puncturing of the UC blood vessels, mixed with ACD anticoagulant and transported (230C) to the cord blood bank laboratory. The average volume of CB was 92 ml, transportation time <24h. Mononuclear cells were isolated in Sepax (BioSafe) separator, and frozen after suspending in 5% human albumin/10% DMSO in controlled-rate freezer (Ice Cube, Sylab). The novel method of UCSC banking was based on freezing down and storage whole fragments of UC Wharton jelly, and UCSC expansion "on demand" after thawing. This method allows to avoid the cost- and time-consuming procedure of cell expansion prior to freezing down, and allows for recovery and expansion of UCSC from over 85% of frozen down Wharton jelly samples. UC was collected under sterile conditions, and transported to the processing laboratory in buffered salt solution (PBS) supplemented with penicillin/streptomycin. Cord was cut into 5 cm-long pieces, and after longitudinal dissection, blood vessels were removed. Resulting Wharton jelly tissue was cut into 5 cm long and 3 mm thick strips, saturated with 5% albumin/10% DMSO,