reproduction techniques (ART) may increase the basal rate of DNA damage. Altogether, this may produce a reduction in the expected fertility rates. Unfortunately, both in freshly ejaculated and in cryopreserved samples, the mechanisms and processes involved in Sperm DNA Fragmentation (SDF) are not fully illuminated. In mammalian species it is known that SDF, far to be a static concept, an increase in the rate of Sperm DNA Fragmentation (rSDF: increase of SDF values through time before fertilization) is produced. Thus, the dynamic behaviour of SDF, more than the static view of this parameter, could be a key factor to understand the potential capacity of male to produce a normal pregnancy. Within the general tendency reported by other authors on different seminal characteristics after sperm capacitation, the most parsimonious hypothesis is that the values of SDF would be improved after capacitation. The aim of this investigation was to analyze the increase of SDF values over time in donors with proven fertility, using an experimental model which emulates the steps of sperm handling as used for ART, using fresh and frozen-thawed and capacitated samples. A dynamic approach of SDF was used to assess such hypothesis.
Material & Methods: SDF assessment was performed in fresh and frozen-thawed and capacitated samples (density gradient isolation) from the same individual after 0.5, 1.5, 4.5, 6, 24, 48 and 72 hours of incubation in a humidified atmosphere of 5% CO_2 in air at 37°C. Study was performed using 15 male donors with proven fertility for a maximum of six births at the reproductive medicine center. SDF fragmentation was assessed under fluorescence microscopy and image analysis software using Halosperm, the commercial variant of the sperm chromatin dispersion methodology.
Results: 1) No significant differences in SDF were obtained when fresh and frozen–thawed and capacitated sperm at a basal time were compared. 2) The rSDF showed by both samples is different and capacitation selects for sperm subpopulations which exhibit a lower range of variance for SDF. 3) Gradient-isolated human sperm capacitated after frozen-thawing select for sperm populations with a more stable rate of SDF than that observed in freshly ejaculated semen samples. 3) Capacitation does not select for the best levels for DNA damage in all individuals when compared with the values and rSDF in fresh semen samples.
Conclusion: Dynamic assessment of SDF offers additional and different information to that obtained from a static viewpoint. In the particular case of this experiment, it is concluded that cryopreservation of sperm samples does not affect the basal level of DNA damage observed in each individual when compared to those obtained in fresh samples. Interestingly, sperm capacitation after thawing renders individuaĺs semen samples more predictable in their dynamic behaviour for an increasing rSDF than the rates observed in fresh semen samples. Sperm selection is not providing the best levels of SDF after capacitation for all individuals. Within the field of DNA damage, we are imperatively called to select the most efficient semen samples by minimizing the severity of DNA damage, for example, being scientifically conscientious of the timing for using the sperm samples in ART according to the dynamic behavior that each sperm sample presents. It is therefore recommended that chromatin changes through time might be used as an additional parameter for the assessment of sperm quality after capacitation or in every circumstance of semen handling for ART.

O-218 Oral Damage to human sperm DNA during cryopreservation is predominantly mediated by oxidative stress

L.K. Thomson[1], S.D. Fleming[2], R.J. Aitken[3], G.N. De Iuliis[3], J. Zieschang[1], A.M. Clark[1]
[1]Fertility First, Centre for Reproductive Health, Hurstville, Australia, [2]Westmead Hospital, Obstetrics and Gynaecology, Westmead, Australia, [3]University of Newcastle, ARC Centre of Excellence in Biotechnology and Development and Discipline of Biological Sciences, Callaghan, Australia

Introduction: Sperm DNA damage is associated with a reduced full-term pregnancy rate and may increase the risk of transmitting genetic mutations to offspring. While studies have revealed that the cryopreservation of human semen generates and exacerbates sperm DNA fragmentation, the mechanisms involved in this type of cryoinjury are largely unknown. Elucidation of these mechanisms may provide insight into preventing such damage and it was therefore the aim of this study to determine the involvement of oxidative stress and apoptosis in this phenomenon.
Materials & Methods: We obtained 60 semen samples from 60 men presenting for assessment of fertility and conducted experiments to determine the cause of cryopreservation-induced DNA fragmentation. The percentage sperm DNA fragmentation was determined using the terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick-end labelling (TUNEL) assay (1, 2), the DNA base lesion 8-hydroxy-2′-deoxyguanosine (8OHdG) was used as the biomarker of oxidative stress and the percentage of caspase positive sperm cells was used as an indicator of apoptosis. In addition, the oestrogenic compound and potential antioxidant genistein, and the caspase inhibitor Z-VAD(OMe)-FMK were used to supplement the cryoprotectant media.
Results: Cryopreservation of human semen led to a significant increase in percentage sperm DNA fragmentation, percentage 8OHdG and percentage caspase positive cells ($P < 0.001$). Percentage DNA fragmentation was highly positively correlated with percentage 8OHdG before ($r = 0.756$, $P < 0.001$) and after cryopreservation ($r = 0.528$, $P = 0.017$). Percentage sperm DNA fragmentation was also positively correlated with percentage caspase positive cells before cryopreservation ($r = 0.711$, $P < 0.001$) but these two parameters were negatively correlated post-thaw ($r = -0.455$, $P = 0.044$). The addition of 50 μM and 100 μM genistein to the cryoprotectant had a significant protective effect on sperm DNA ($P < 0.001$) while the caspase inhibitor demonstrated no difference to the control.
Conclusions: Sperm DNA fragmentation is associated with apoptosis and oxidative stress in fresh semen and these two pathways leading to sperm DNA fragmentation appear to act concurrently. An increase in oxidative stress occurs during cryopreservation, which is responsible for the observed increase in DNA fragmentation and 8OHdG. The addition of the oestrogenic compound genistein or a similar antioxidant should prove to be an invaluable addition to the cryoprotectant media currently available and such an improvement could go far in reducing the risks of human sperm cryopreservation but further independent studies will be necessary to confirm our findings.

Thomson et al., (2009a) The DNA integrity of cryopreserved spermatozoa separated for use in assisted reproductive technology is unaffected by the type of cryoprotectant used but is related to the DNA integrity of the fresh separated preparation. Fertil. Steril. (in press).
Thomson et al., (2009b) The effect of repeated freezing and thawing on human sperm DNA fragmentation. Fertil. Steril. (in press).

O-219 Oral Activity of maturation promoting factor, but not of microtubule-activated protein kinase, decreases over time in frozen thawed human oocytes

A. Borini[1], L. Bogliolo[2], G. Coticchio[1], V. Bianchi[1], A. Nicoli[3], G. La Sala[3], G.G. Leoni[4], B. Valli[3], S. Ledda[2]
[1]Tecnobios Procreazione, Centre for Reproductive Health, Bologna, Italy, [2]University of Sassari, Department of Pathology and Veterinary Clinic, Sassari, Italy, [3]Arcispedale S. Maria Nuova, Department of Obstetrics and Gynecology, Reggio Emilia, Italy, [4]University of Sassari, Department of Physiological Biochemical Cellular Sciences, Sassari, Italy

Introduction: Following cryopreservation, the developmental ability of the human oocyte may be affected by diverse forms of cell damage, including perturbances in the level of regulatory proteins. In this study, in frozen-thawed human mature oocytes, we examined the biochemical activity of maturation promoting factor (MPF) and microtubule-activated protein kinase (MAPK), two key regulators of the meiotic and mitotic cell cycles that ensure meiotic arrest, normal spindle configuration and chromosome condensation at the metaphase II (MII) stage.
Material & Methods: Surplus human oocytes were donated by consenting IVF patients. Following cumulus cells removal, oocytes showing normal morphology and an extruded first polar body were selected, and either placed in 2 μL of ice-cold collection buffer (1 mg/mL PVA, 5 mM EDTA, 10 mM Na3VO4, 10 mM NaF) and stored at −80°C, or cryopreserved by slow cooling. Cryopreservation involved exposure to 1.5 mol/l propanediol and 0.2 mol/L sucrose before freezing, while after thawing cryoprotectant dilution was performed in the presence of 0.3 mol/L sucrose and decreasing concentrations of propanediol. Before placement in ice cold buffer and storage at –80°C, frozen-thawed samples were cultured for 1 or 2 hours. To perform the MPF and MAPK assays, fresh and cryopreserved samples were recovered from −80°C storage and brought to a final volume of 9 μL with a solution containing 45 mM β-glycerophosphate, 12 mM p-nitrophenylphosphate, 20 mM

MOPS-KOH, 12 mM MgCl2, 12 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 2.3 mM NaVO4, 2 mM NaF, 0.8 mM PMSF, 15 μg/mL leupeptin, 30 μg/mL aprotinin, 0.1% (w/v) PVA, 1 mg/mL histone H1 (type III-S from calf thymus), myelin basic protein (1 mg/mL, MBP), 2.2 M protein kinase inhibitor peptide, and 2.5 MBq/mL γ-[32P]-ATP. The reaction was started after the addition of γ-[32P]-ATP and performed for 30 min at 37°C. Phosphorylations of substrates histone H1 and MBP where considered as signs of MPF and MAPK activity, respectively. Proteins were separated on 1D SDS-PAGE electrophoresis and radioactive bands were analyzed by gel autoradiography. The pixel intensity of a preselected set area was measured using Kodak Image Analysis Software 1D 3.6. The mean band intensity of fresh controls was assumed to correspond to 100 arbitrary units and the mean band intensities of the other groups were quantified comparatively to this value. Analysis of variance (ANOVA) was used to assess the significance of differences in MPF and MAPK activity among groups. $P < 0.05$ was considered significant.

Results: In frozen-thawed oocytes that where cultured for 1 hour after thawing, the relative intensity (RI) of the MPF bands was not statistically different (98.2) from the fresh control. However, MPF band intensity was significantly reduced ($RI = 73.3$, $P < 0.05$) in samples cultured for 2 hours after freezing-thawing. Conversely, comparable intensities of the MAPK bands were observed in fresh or cryopreserved oocytes cultured for 1 or 2 hours ($RI = 100$, 96.8, and 98.7, respectively).

Conclusions: In human oocytes, slow-freezing appears to partly influence the activity of cell cycle regulatory proteins. After thawing, MAPK is unaffected over a period of two hours. During the same interval, MPF activity is initially maintained unaltered but undergoes a significant decrease thereafter. This may have significant implications for the use of frozen-thawed oocytes. In particular, considering the observed delayed reduction in MPF activity, it might be appropriate to limit to 1 hour the post-thaw period in which oocytes are cultured before sperm microinjection, thereby preventing possible losses in spindle and chromosome configuration, or premature exit from the MII arrest.

O-220 Oral Cryoleaf vitrification increases the rate of chromosome misalignment in the metaphase II spindle of human mature oocytes

G. Coticchio[1], J. Bromfield[2], R. Sciajno[1], A. Gambardella[1], A. Borini[1], D.F. Albertini[2]
[1]Tecnobios Procreazione, Centre for Reproductive Health, Bologna, Italy, [2]University of Kansas Medical Centre, Department of Molecular and Integrative Physiology, Kansas City, U.S.A.

Introduction: Following cryopreservation, the metaphase II (MII) spindle of the human oocyte may be damaged from non-physiological depolymerization or loss of organization as a consequence of its susceptibility to low temperatures and physical stress. This may predispose the oocyte to errors in chromosome segregation during meiosis II, fertilization failure and cleavage anomalies. Several studies on the MII spindle have involved oocytes stored with slow cooling protocols, but evidence on vitrified oocytes is still scarce. In the present study, using confocal microscopy, we assessed meiotic spindle organization and chromosome alignment in oocytes after vitrification by the cryoleaf system.

Material and methods: Surplus human oocytes were donated by consenting IVF patients. Three hours after retrieval, following cumulus cells removal, oocytes showing normal morphology and an extruded first polar body were selected and immediately fixed ($n = 22$) or vitrified ($n = 43$). Vitrification and warming solutions were prepared in TCM199. Oocytes were equilibrated for 15 minutes at room temperature (RT) in equilibration solution containing 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO). Afterward, they were placed for 1 minute in vitrification solution, consisting of 15% EG, 15% DMSO and 0.5 mol/l sucrose, loaded onto cryoleaf strips, and immediately submerged into liquid nitrogen (LN2). Warming was conducted by direct transfer from LN2 to thawing solution (1.0 M sucrose), at 37°C. After 1 minute, oocytes were placed for 3 minutes at RT in dilution solution, including 0.5 M sucrose, washed (5 min) twice in TCM199, and cultured for 1 hour. Oocytes were fixed using a mixture containing taxol, formaldehyde, and triton, and labelled for total tubulin (anti-αβ tubulin cocktail) and chromatin (Hoechst 33258). Oocytes were analysed using a Zeiss LSM Pascal confocal imaging system. A complete z-series for each spindle was collected at 0.7 μm intervals and reconstructed as a rendered 3-dimensional image. Spindles were classified according to shape, length and polar constriction. Chromosomes were classified as scattered or aligned at the metaphase plate. A P value <0.05 was considered statistically significant.

Results: Tubulin staining was found in all vitrified/warmed (v/ w) oocytes, the majority (17/43, 62.8%) of which displayed a bipolar spindle with focussed poles. However, a normal bipolar spindle configuration and equatorial chromosome alignment was observed only in 32.6% of all v/w oocytes (14/43). This frequency was significantly lower in comparison to the fresh group (59.1%). In another fraction of v/w oocytes (13/43, 30.2%), spindle bipolarity was associated to one or more non-aligned, scattered chromosomes. In such a case, chromosomes often appeared to jut out from the spindle outline. In 30.2% (13/43) of v/w oocytes, chromosome alignment was not accompanied by spindle bipolarity, while in a small minority (3/43, 70%) both microtubules fibers and chromosomes were disarranged. Furthermore, in cryopreserved oocytes with a bipolar spindle a tendency towards an increased pole-to-pole distance (14.9 ± 2.3 μm) was found in comparison to the fresh control (12.4 ± 2.6 μm).

Conclusions: Under the conditions tested, in the majority of vitrified oocytes the MII spindle maintains a bipolar organization. However, chromosome alignment appears to be partly compromised. In addition, a certain proportion v/w oocytes shows rather atypical features, i.e. elongated spindles and protruding chromosomes. This might predispose v/w oocytes to an increased risk of meiotic errors. Further studies are in progress to ascertain whether the observed anomalies are recoverable during post-warm culture, and therefore only transiently expressed, or persist after warming.

O-221 Oral Oocyte cryopreservation is an efficient alternative to embryo freezing

M.G. Minasi[1], M. Iacobelli[1], V. Casciani[1], A. Colasante[1], P. Rubino[1], F. Scarselli[1], S. Ferrero[1], K. Litwicka[1], Z.P. Nagy[2], E. Greco[1]
[1]European Hospital, Reproductive Medicine, Rome, Italy, [2]Reproductive Biology Associates, Reproductive Medicine, Atlanta Georgia, U.S.A.

Introduction: Italian law, introduced in 2004, imposes that no more than three oocytes can be fertilized and the same law prohibits embryo cryopreservation. Thawing and transfer of the embryos frozen prior to this law is still allowed. Oocytes cryopreservation is permitted, which currently is the only alternative to embryo freezing. Efficiency of oocyte cryopreservation has been significantly lower than embryo freezing, however modified protocols have resulted in improved outcomes. The aim of this study is to evaluate if the oocyte cryopreservation can serve as a viable alternative to embryo freezing in terms of clinical outcomes.

Materials and Methods: Study period was between March 2004 and December 2008. Patient inclusion criteria: 1) female age < 39 years (at the time of the oocyte collection) and 2) cryopreserved oocytes or embryos. Group A: transfer of frozen/thawed embryos and Group B: transfer of embryos obtained from cryopreserved oocytes. Excellent and good quality supernumerary embryos were frozen using slow freezing protocol with 1.5 M propandiol (PROH) and 0.1 M sucrose. Good quality supernumerary oocytes were frozen using three different protocols: 1) slow freezing with 1.5 M PROH and 0.1 M sucrose; 2) vitrification on gold grids with 1.5 M and 5.5 M ethylene glycol (EG) and 1 M sucrose; 3) vitrification on cryotops with 1.33 M (7.5%) and 2.66 M (15%) EG, 1.06 M (7.5%) and 2.12 (15%) dimethyl sulfoxide (DMSO) and 0.5 M sucrose. Slow-frozen embryos and oocytes were thawed using in 1.0, 0.5 and 0.0 M PROH and 0.1 M sucrose. Vitrified oocytes were warmed with sucrose 1 M, 0.5 M, 0.25 M, 0.125 M and 0 M (for gold grids) or 1 M, 0.5 M and 0.0 M (for cryotops). Statistical analysis were performed using Fisher's exact and Mann-Whitney tests at the level of $P \leq 0.05$.

Results: Groups A and B include 207 and 125 thawing/warming cycles, respectively. Mean female age ± SE at the day of the freezing was not statistically different between the two groups (33.1 ± 0.21 in Group A and 33.4 ± 0.29 in Group B). In Group A, 678 embryos were thawed and 454 of them survived (66.9%) whereas in Group B, 343 out of 849 thawed/warmed oocytes survived (40.4%, $P \leq 0.001$). More patients had embryo-transfer in Group A (88.9%, $N = 193$) as compared to Group B (64.8%, $N = 81$, $P \leq 0.001$). Embryos transferred were 449 in Group A and 176 in Group B. There were not significant differences in pregnancy rate per transfer [28.5% of patients ($N = 55$) in Group A and 29.6% ($N = 24$) in Group B], in implantation rate [8.0% of embryos ($N = 449$) in Group A and 11.9% ($N = 176$) in Group B] and in