

have significantly increased over the SF blastocysts. Since we transferred an average of fewer V blastocysts per cycle and 50% of our pregnancies resulted in twins, we are aiming at single embryo transfer of the V day 5 blastocysts in the future.

P-77

Cryopreservation of supernumerary day-3 embryos with poor quality: Is there any benefit for extending culture to blastocyst before freezing?
Navid Esfandiari, Robert F. Casper, Sergey Moskovtsev, Murid Javed, Elizabeth A. Claessens, Lynda Gottlieb. Toronto Ctr for Advanced Reproductive Technology, Toronto, ON, Canada.

Objective: Although cryopreservation of supernumerary embryos has increased the overall success rate in human IVF-ET, the survival and implantation rate of frozen-thawed embryos is directly related to the quality of embryos at the time of freezing. Theoretically, extending culture to blastocyst for poor quality embryos will allow the embryologist to select and freeze the viable embryos with potential for implantation. The objective of our study was to determine the value of extended culture to blastocyst stage of supernumerary slow (< 6-cell on day-3) and poor quality embryos (grade $\geq$ III) that seemed unsuitable for freezing on day-3.

Design: Retrospective analysis of 159 poor quality day-3 embryos from 43 treatment cycles between October 2001 and August 2002 in a tertiary care facility.

Materials and Methods: Oocytes were inseminated in human tubal fluid (HTF) + 10% synthetic serum substitute (SSS). Injected oocytes and normal zygotes (2PN) were cultured in 50 mL drops of either HTF-SSS or in G1 media under oil. Embryo quality on day-3 was determined according to morphologic features including blastomere number, evenness of blastomeres and the presence and extent of fragmentation. Cleavage stage embryos were graded from 1 to 5 with grade 1 representing the best quality embryos. Embryos with the best morphology were chosen for fresh transfer or cryopreservation. For all other remaining slow (< 6-cell) or poor quality embryos derived from IVF or ICSI, culture was extended to blastocyst (using Irvine blastocyst or G2 culture media, respectively). Embryos were maintained individually in culture up to day-6 to track daily growth until blastocyst formation or arrest.

Results: Of the 159 slow or poor quality day-3 embryos undergoing extended culture, 28 formed blastocysts (17.6 %) either on day-5 or day-6 of culture. There was no significant difference in blastocyst formation between the embryos cultured in Irvine blastocyst media or G2 media ($p > 0.05$). Blastocyst formation took place for both slow and low-grade embryos, although none of the 3-cell day-3 embryos ($n = 13$) developed to blastocyst (Table I). The overall blastocyst development rate (BDR) for slow and poor quality embryos were 13.5% and 33.3%, respectively.

Table 1. Blastocyst development rate in poor quality and slow day-3 embryos

Day-3 Embryos	Blastocysts		
	Day-5	Day-6	Total
4 and 5-cell (GI, II)	8.3% (7 / 84)	6 % (5 / 84)	14.3% (12 / 84)
4 and 5-cell (GIII, IV)	5 % (1 / 20)	5 % (1 / 20)	10% (2 / 20)
6 to 8-cell (GIII, IV)	14.3 % (6 / 42)	19 % (8 / 42)	33.3% (14 / 42)

Conclusion: The results of our study have shown that extended culture of slow and poor quality embryos resulted in significant blastocyst development, therefore they should be considered as viable and valuable embryos either for fresh transfer or freezing. However, extended culture of embryos for 3 to 4 more days requires extra laboratory activity, observation, and expense, and since blastocysts have a lower thaw survival rate than cellular stage embryos, our results suggest freezing the poor quality 6 to 8-cell and slow embryos with normal morphology on day-3, and transfer or culture surviving embryos to blastocyst after thawing.

P-78

Sperm glutathione antioxidant defense system protects from cryodamage after a cycle of freezing and thawing. Marcos Meseguer, Nicolas

Garrido, Manuel Rodriguez, Carlos Simon, Antonio Pellicer, Jose Remohi. Inst Valenciano de Infertilidad, Valencia, Spain.

Introduction: Freezing and subsequent thawing of spermatozoa affects the percentage of alive and motile cells together with their fertilizing ability. Oxidative stress is a relevant phenomenon affecting sperm properties. Then, an oxidative stress imbalance can be predisposing to freezing damage. This situation is counteracted with different oxidative defense enzymes and antioxidants such as glutathione peroxidases -1 and -4 (GPxs), glutathione reductase (GR) and cellular glutathione (GSH). Thus, the oxidative status could be a predictive marker of sperm quality after freezing-thawing process. Our aim with this study was to determine the ability of determining mRNA levels and enzymatic activity of the above mentioned molecules as thawing predictors.

Methods: This study was performed in 56 human sperm samples undergoing a freezing-thawing process, samples were studied according with WHO statements for concentration of motile and alive sperm both freshly and after thawing. We measured gene expression of GPx-1 and -4 and GR by quantitative fluorescent polymerase chain reaction (qfPCR) by using LightCycler-FastStart DNA Master SYBR green I (Roche Molecular Biochemicals). Cellular glutathione (GSH) concentrations and GPxs and GR activities were measured by spectrophotometry under controlled biochemical reactions. Quantity of protein in the sperm samples was normalized with Bradford's method. One way analysis of variance was used to detect differences between parameters studied and sperm rate survival after thawing. Linear regression analysis was used to determine the extent of correlation between parameters compared. The diagnostic performance of the tests performed is evaluated using Receiver Operating Characteristic (ROC) curve analysis.

Results: In the following table results are summarized, mRNA is expressed as fluorescence arbitrary units, activity is expressed as nmol of NADPH consumed by mg of protein and minute and GSH concentrations in μ M. AUC^{ROC}: area under the curve in a receiver operating characteristic analysis. CI: confidence interval. PPV: positive predictive value. NPV: negative predictive value.

We observed a direct and statistically significant ($p < 0.05$) relationship between GPxs expression and activity and GSH concentrations, respectively with post-thaw motile sperm recovery rate (PTRR) but not with GR and any other basic semen parameters. Predictive values clearly demonstrated that better diagnostic accuracies among the molecules analyzed are those for GPx-1 and -4 mRNA expression, and enzymatic activity, and GSH concentration.

Parameters	AUC ^{ROC} (95% CI)	Significance	Threshold	Sensitivity (%)	Specificity (%)	PPV	NPV
GPX1 mRNA	0.82 (0.62-0.94)	0.03 ^a	>12.1	80.0	95.2	80.0	95.2
GPX1 activity	0.74 (0.53-0.88)	0.04 ^a	>445	45.5	94.1	83.3	72.7
GPX4 mRNA	0.94(0.78-0.99)	0.02 ^a	>7.2	80.0	100.0	100.0	95.5
GPX4 activity	0.74 (0.54-0.89)	0.02 ^b	>145	72.7	82.4	72.7	82.4
GR mRNA	0.65 (0.44-0.82)	0.27 ^b	$\leq$ 12.3	83.3	57.1	35.7	92.3
GR activity	0.60 (0.41-0.78)	0.35 ^b	$\leq$ 0.4	58.3	70.6	58.3	70.6
GSH	0.73 (0.52-0.87)	0.04 ^a	$\leq$ 2.1	81.8	70.6	64.3	85.7

Conclusions: Our results clearly implicate GSH system and oxidative stress status of sperm cells before freezing in their characteristics and sensitivity to cryodamage. GSH reserve together with GPx-1, -4 activity eliminating free radicals using GSH is essential for a good post thawing recovery and can be employed as predictors of post-thawing semen quality.

P-79

Prospective randomized comparison between transfer of blastocyst stage embryos and cleavage stage embryos during frozen cycles after ICSI procedure. Amr M. A. Azim, Iqbal Khan, Michael H. Fakih, F. Nicholas Shamma, Jonathan W. T. Ayres, Mustafa I. Abuzeid. Michigan State Univ, Grand Blanc, MI; IVF Michigan, Rochester Hills, MI; Michigan State Univ, IVF Michigan, Rochester Hills, MI; IVF Michigan and Michigan State Univ, Coll of Human Medicine, Rochester Hills, MI.

Objective: To compare pregnancy outcome following transfer of blastocysts or cleavage stage embryos after thawing of frozen zygotes resulting from ICSI cycles.

Design: Prospective nonblinded randomized study.

Materials and Methods: One hundred and forty two consecutive frozen cycles in the period between January, 2000 and December, 2002, were recruited for this study. During fresh cycles, controlled ovarian hyperstimulation was accomplished using gonadotropin releasing hormone agonist or antagonist and recombinant FSH and/or hMG. ICSI procedures were performed on all oocytes. All patients received day 3 cleavage embryos in their fresh cycles. Supernumerary zygotes were frozen. Patients were randomized at the time of initiation of frozen cycles into two groups. In Group I (60 cycles) thawed zygotes were cultured to blastocyst stage. In Group II (82 cycles) thawed zygotes were cultured to cleavage stage embryos. Embryo transfer was cancelled if no blastocysts or cleavage stage embryos suitable for transfer were obtained. Clinical pregnancy and delivery rates of fresh and frozen cycles were calculated. Statistical analysis was performed using student t-test, Chi square analysis and correlation analysis whenever appropriate.

Results: There was no statistically significant difference between the two groups with respect to age, underlying etiology, number of oocytes retrieved, number of zygotes frozen in the fresh cycles and number of day 3 cleavage stage embryos in culture in the frozen cycles. Outcome of frozen cycles is summarized in the table below.

	Group I (Blastocysts)	Group II (cleavage embryos)	p
No of cycles	60	82	
No of cycles with transfers	44	67	
No of embryo transferred / cycle	2.1 ± 0.1	3.2 ± 0.1	NS
Cancellation rate (%)	26.7	18.7	NS
Pregnancy rate per cycle (%)	35.5	18.3	<0.025
Ongoing / delivery rate per cycle	33.3	17.1	<0.05
Pregnancy rate per embryo transfer (%)	45.5	22.4	<0.01
Ongoing / delivery rate per embryo transfer (%)	43.2	20.9	<0.01

There was no significant difference in miscarriage rate, ectopic pregnancy rate and multiple pregnancy rate between the two groups. In addition, there was no correlation between the number of pregnancies in frozen cycles and the number of oocytes retrieved or the number of zygotes frozen. Furthermore, there was no correlation between the occurrence of pregnancy in the fresh and the frozen cycles.

Conclusions: This data suggest that during frozen cycles of zygotes resulting from ICSI procedures, blastocyst stage embryo transfer could achieve higher pregnancy and delivery rates compared to cleavage stage embryo transfer.

P-80

Improved outcome of frozen-thawed blastocyst transfer with Menezo's 2-step thawing protocol. Jianchi Ding, Mingxian Shen, Nasir Rana, W. Paul Dmowski. Oak Brook Fertility Ctr, Oak Brook, IL.

Objective: To compare the effect of two different thawing protocols on cryopreserved blastocyst recovery rate and pregnancy outcome after transfer.

Design: Retrospective analysis.

Materials and Methods: 94 consecutive frozen-thawed blastocyst transfer (FBT) cycles from 1996 to 2002 were analyzed. All blastocysts were cryopreserved using Menezo's 2-step slow freezing protocol (media prepared in house) on day-5 or day-6 (egg retrieval=day-0). Blastocysts were thawed for transfer using either Menezo's stepwise thawing protocol (59 cycles, media prepared in house) or 2-step protocol (35 cycles, media from Irvine Scientific, Santa Ana, CA). Immediately after thawing, assisted hatching was performed on all embryos. All embryos were then cultured in blastocyst culture medium for 3 to 5 hours before transfer and were classified into 4 categories: 1) Re-expanded: blastocysts recovered >80% of the original size with a single blastocoele; 2) Re-expanding: blastocysts recovered <80% of the original size with clear blastocoele; 3) Non-expanding: having more than 50% live cells, but no evidence of re-expansion and 4) Non-viable: with less than 50% live cells and with no signs of re-expansion. Only viable embryos were selected for transfer. After transfer, patients showing β -hCG elevation were defined as β -hCG positive; those

with intrauterine gestational sac(s) were defined as clinical pregnancies (Clinical PR); those with fetal cardiac activity as ongoing pregnancies (Ongoing PR); and those who delivered live babies as live births. Patients who had β -hCG elevation but no gestational sac were considered as biochemical pregnancies (Biochem PR). The data were analyzed using χ^2 or student t-test where appropriate. A p value of < 0.05 was considered significant.

Results: The percentage of re-expanded blastocysts was similar for 2-step and stepwise thawing (42.9% vs 46.8%, $P>0.05$); the proportion of re-expanding blastocysts was lower in 2-step thawing than in stepwise thawing, but not statistically significant (26.7% vs 34.5%, $P>0.05$); and the percentage of non-expanding blastocysts was significantly higher in 2-step thawing than in stepwise thawing (30.5% vs 18.7%, $P<0.05$). Embryo transfers were performed in all 35 FBT cycles after 2-step thawing and in 57 of 59 FBT cycles after stepwise thawing. Mean number of embryos transferred per cycle was similar in both groups (2.74 and 2.88 for 2-step and stepwise thawing, respectively; $P>0.05$). Implantation rate, ongoing pregnancy (determined by the presence of fetal cardiac activity) and live birth rates were significantly higher with 2-step (25%, 45.7% and 42.9%) than with stepwise thawing protocol (9.2%, 18.6% and 16.9%, $P<0.01$). The miscarriage rate (including biochemical PR) was lower with 2-step thawing than with stepwise thawing (28.6% vs 50%), but not statistically significant ($P=0.15$). Furthermore, the percentage of gestational sacs resulting in live babies was higher with 2-step thawing than with stepwise thawing (76.6% vs 50%, $P=0.04$).

Conclusion: We conclude that the 2-step thawing is a better thawing protocol when compared to the stepwise thawing method. The 2-step thawing protocol dramatically increased the embryo implantation potential and resulted in higher pregnancy rate after frozen blastocyst transfer even though the recovery rate of thawed blastocysts with the 2-step thawing was somewhat lower than with the stepwise thawing protocol.

P-81

Optimization of a thawing method for human expanded blastocysts vitrified using EM-Grid after artificial shrinkage. So-Young Lee, Byung-Suk Jae, Seok-Yoon Lee, Weon-Young Son, San-Hyun Yoon, Jin-Ho Lim. Maria Infertility Hosp, Seoul, Republic of Korea.

Objective: We have achieved the high survival and acceptable pregnancy rates with artificial shrinkage (i.e. induced collapse of the blastocoele) and 6-step thawing in cryopreservation of human expanded blastocysts (Cho et al., 2002; Son et al., 2003). The purpose of this study was to compare the survival rates of human expanded blastocysts thawed by different methods after vitrification following artificial shrinkage and to report the successful pregnancies and live births from the transfer of blastocysts survived by the established thawing method.

Design: Establishment of simple thawing method which is appropriate for human expanded blastocysts vitrified after artificial shrinkage and clinical application of the method

Materials and Methods: After ET in IVF cycles, surplus expanded blastocysts ($>160 \mu\text{m}$) ($n = 279$) that were not suitable to be frozen were donated from patients. After artificial shrinkage, the blastocysts were equilibrated in 20% ethylene glycol for 1.5 min, exposed to solution containing 40% ethylene glycol, 18% Ficoll and 0.3 M sucrose, loaded onto the EM-grid, and then directly plunged in liquid nitrogen. After thawing, cryoprotectants were diluted in 0.5 M sucrose for 3, 4, 5 or 6 min, respectively. The survival rate of the blastocysts was compared each other. In addition, the method established from above result was compared to 6-step thawing method (0.5 M sucrose for 3 min, in turn, 0.4, 0.3, 0.2, 0.1 and 0 M of sucrose at intervals of 1.5 min). The established 2-step method was applied clinically to patients ($n = 85$) who had participated in our thawing program from March 1, 2002 to March 31, 2003.

Results: The survival rate of blastocysts (86.7%, 26/30) diluted in 0.5 M sucrose for 5 min after thawing was higher than those of 3 min (60.9%, 14/23), 4 min (79.2%, 19/24) and 6 min (76.0%, 19/25). There was no difference between the 6-step method (87.4%, 76/87) and an established 2-step method (0.5 M sucrose for 5 min) (85.6%, 77/90) in survival rate of blastocysts. The resulting clinical pregnancies was 49.4% (42/85) and 10 live births were achieved in the present.

Conclusion: The present result indicates that the 2-step thawing method, which incubates thawed blastocysts in 0.5 M sucrose for 5 min, should be