Abstracts Presented at the 2012 CRB Symposium

held during AAB's Annual Meeting and Educational Conference

2012 Abstracts

AMH Cutoff Level Predicting Clinical Pregnancy Following IVF In Women With Diminished Ovarian Reserve

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Objective: Women diagnosed with diminished ovarian reserve (DOR) by virtue of age and/or FSH can be further subcategorized by AMH level. AMH levels of <0.8 ng/mL are used clinically in our practice to confirm diagnosis of severe DOR. Once age, FSH, AMH and antral follicle count (AFC) are evaluated, the DOR patient is counseled on their chances for IVF success.

Hypothesis: IVF cycle response and clinical pregnancy (CP) of women with DOR and AMH levels of <0.8 ng/mL are significantly worse than that of women with DOR and AMH levels ≥0.8 ng/mL.

Materials and Methods: All DOR patients (ages ≥35) with a historical high FSH of >10 ng/mL who had available AMH levels and underwent IVF/ICSI cycles at Montefiore's Institute for Reproductive Medicine and Health between January 2008 and December 2011 were included in this retrospective study (n=85). DOR patients were categorized into three groups: AMH <0.2 ng/mL (Group 1, n=27), AMH =0.2 - 0.79 ng/mL (Group 2, n=41) and AMH ≥0.8 ng/mL (Group 3, n=17). Data collection included patient age, BMI, cycle day 3 FSH and E2, AFC, total gonadotropins dose, cycle cancellation rate, # eggs retrieved, # mature eggs, and CP per cycle start. ANOVA, Kruskal Wallis and chi square tests with post hoc analysis were used as appropriate for comparison among the three groups. P value <0.05 was considered to be statistically significant.

Results: Cycle stimulation and outcome parameters for the three groups are shown in the table below. Group 3 had significantly more oocytes retrieved and more mature oocytes than Groups 1 or 2 (p=0.01 and p=0.001, respectively). Group 2 and 3 had significantly higher CP rates per cycle start compared to Group 1 (p=0.04). Interestingly, although Group 2 had significantly fewer eggs retrieved and mature eggs than Group 3, CP rates per cycle start for the two groups were not different.

Conclusion: AMH of ≥0.2 ng/mL appears to be a meaningful threshold for predicting CP in women with severe DOR at our practice. This information is crucial during the pre-cycle counseling of these women.

Parameter	Group 1 AMH <0.2 ng/mL	Group 2 AMH =0.2 – 0.79 ng/mL	Group 3 AMH ≥0.8 ng/mL	p-value
Age (yr)	41.2 ±3.7	39.3 ±3.1	40.3 ±2.7	0.34
BMI (kg/m²)	25.8 ±5.7	25.9 ±4.9	25.8 ±3.9	0.26
AFC (#)	5.1 ±2.0	7.3 ±2.6	7.6 ±2.9	0.18
Day 3 FSH (IU/l)	11.2 ±4.3	9.6 ±3.5	9.4 ±2.5	0.07
Day 3 E2 (pg/mL)	44.5 ±23.1	39.7 ±18.0	40.2 ±15.1	0.18
Gonadotropin dose (IU)	4667.4 ±1851.5	4592.2 ±1852.3	4598.4 ±1595.6	0.77
Cycle cancellation	12 (44)	15 (36)	3 (18)	0.18
ICSI	12 (80)	25 (96)	13 (93)	0.26
AZH	12 (80)	17 (65)	12 (86)	0.49
# oocytes retrieved	**3.80 ±2.2**	**5.07 ±2.1**	**8.40 ±3.9**	**0.01***
# mature oocytes	**2.86 ±2.0**	**4.0 ±2.2**	**6.57 ±3.1**	**0.001***
# embryos transferred	1.4 ±1.0	2.07 ±1.3	2.43 ±1.8	0.09
CP per cycle start	**1 (4)**	**11 (27)**	**3 (18)**	**0.04***

Data are expressed as means ± SD or n (%)

Are Metaphase I (MI) Oocytes Worth Injecting At The Time Of ICSI?

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Introduction:
In the fully developed antral follicle of the mammalian ovary oocytes are arrested in meiotic prophase, but they are fully competent to resume meiosis. The LH surge is the signal that triggers oocyte reentry into the cell cycle prior to ovulation. In ART practice the oocytes are aspirated from the follicles before ovulation occurs and are usually collected at various stages of meiotic maturity. MI oocytes have already undergone germinal vesicle breakdown (GVBD) and are capable of extruding the first polar body (PB) within hours after egg retrieval [1]. Previous reports have shown that pregnancies are rare after ICSI on oocytes

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that started out as MI's [2, 3, 4]. This study analyzes the results of ICSI performed on MI stage oocytes over a period of 2 years and takes genetic information into consideration when available.

Objective:
The goal of this study was to analyze the results from two years of ICSI performed on MI oocytes and evaluate their clinical significance.

Materials and Methods:
Denuding of cumulus cells from the oocyte was performed as routine between 1 and 2 hours after egg retrieval. ICSI was performed between 4 and 5 hours after egg retrieval and all MI oocytes were injected regardless of whether they had extruded their first PB or not by the time of ICSI. No distinction was made in this analysis for sperm source (ejaculate vs. surgical retrieval) or use of oocytes from young donors. All the Day 3 biopsies were performed in-house and all aneuploidy screening was performed by NATERA (formerly Gene Security Network).

Results:
This study looked at 836 cycles that had ICSI as the fertilization method, from January 2010 through December 2011. Our experience was that ~11% of the retrieved oocytes were at the MI stage, ~11% were at the germinal vesicle (GV) stage and ~78% were at the metaphase-II (MII) stage. In agreement with what was previously reported [2, 3, 4] MI injected oocytes gave a lower fertilization rate (% of 2PNs) compared with siblings MII oocytes (31% vs. 70%, $p<0.01$). Moreover, MI oocytes showed a significant difference in blastocyst formation rate on the morning of Day 5 (27% vs. 48%, $p<0.05$) and in the overall percentage of usage (31% vs. 45%, $p<0.01$) compared with siblings MII oocytes. Embryos coming from fertilized MI oocytes (MI-embryo) were transferred in 54 cycles together with other embryos coming from fertilized MII oocytes (MII-embryo) and in 5 cycles, only MI-embryos were transferred. Implantation rates were 14%, 20% and 46% for cycles where mixed embryos, MI-embryos only or MII-embryos only were transferred. Interestingly, one healthy baby was born from a Day 2 transfer of an embryo that originated from an MI egg that had extruded the first PB at time of ICSI (IVM-MI).

Among the patients studied here, embryos from 47 cycles underwent Day 3 blastomere biopsy for aneuploidy screening. A total of 598 MII-embryos were biopsied and the overall euploidy rate for these embryos was 30.4% with an average patient age of 35. In addition to these 47 cycles, embryos from 11 more cycles were biopsied where MI-embryos were biopsied along with their MII-embryo siblings. Sixteen MI-embryos were screened together with 118 MII-embryos. The results of the analysis for these embryos showed that the euploidy rate was significantly higher in the group of MII-embryos compared to their MI-embryo siblings (32.2% vs. 0%), and not different from the results of the 47 cycles where only MII- embryos were analyzed.

Discussion:
A clinical benefit from the ICSI of immature oocytes has not been established and their use is often limited to cases of poor responders or patients with a very unsynchronized cohorts of follicles. Literature on MI oocytes is slowly accumulating and the consensus is that injection of the MI oocyte after extrusion of the PB (in vitro matured; IVM-MI) gives better results than the injection of MI oocytes prior to the extrusion the first PB [3]. Nevertheless, there seems to be no agreement regarding the optimal incubation period for in vitro maturation, and is know that fertilization rates drop when MI oocytes require a longer incubation time to reach the MII stage [5]. For convenience we decided to inject all the MI oocytes together with the MII oocytes at time of ICSI regardless of whether the first PB had appeared or not. This approach yielded results for fertilization and embryo development that are in agreement with the current literature.

The number of pregnancies achieved after the transfer of MI-embryos remains very low and is limited to case reports in the literature. However, the achievement of just one live birth in 2 years and among 836 cycles, makes the use of MI oocytes in ART a practice that may be considered grossly inefficient. In addition, the finding that no embryo created from MI egg had normal chromosome content when analyzed at the cleavage stage may suggest that injecting MI oocytes carries some risk, particularly for patients not undergoing genetic testing. Future studies on results from trophectoderm biopsy are needed to exclude the possibility of wrong call due to the mosaicism effect that is associated with Day 3 embryos. The results suggest that MI oocytes should not be injected with sperm unless they have completed meiosis.

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Clinically Toxic Mineral Oil: Embryos from Outbred Mice Are Sensitive Surrogates for Human Embryos

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Mineral oil is a heterogeneous petroleum product with variable quality. The amount and nature of toxins is not fully understood; however, peroxide activity is one known source of toxicity. Due to limitations of assays for peroxides, the mouse embryo assay (MEA) remains the method of choice for oil toxicity testing. Though all oil sold for use in the clinical IVF laboratory is tested and approved with the one-cell mouse embryo assay, at least 3 lots of oils have been recalled by 3 separate companies over the past 2 years. We have previously shown that embryos from outbred mice are more sensitive to toxins than the standard MEA with embryos from hybrid mice. The purpose of this study was to compare the sensitivity of embryos from outbred (CF1) to embryos from inbred mice (FVB). Two of the affected lots of oil, L1 and L2, were compared with control oil, C. MEA was performed with HTF (In Vitro Care) containing polyvinyl alcohol (PVA). Expanded blastocyst rate at 96 hours was the endpoint. Blastocyst rates for embryos from inbred mice were similar (90±12%, 80±6% and 88±11%) for C, L1 and L2, respectively. In contrast, blastocyst rates for embryos from outbred mice were lower for L1 and L2 (22±10% and 21±12%) vs control oil (56±5%; $P < 0.05$). Further testing using a SafTest® assay revealed elevated levels of peroxides in L1 and L2. These results indicate that oil containing low but clinically significant levels of peroxide can be detected with one-cell embryos from outbred mice.

Effect Of Chromosomal Aneuploidy On Early Embryonic Development In Vitro

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Objective: In clinical IVF, following successful fertilization in-vitro, many embryos fail to develop to the blastocyst stage. This may be due to chromosomal aneuploidy. This study analyzes the relationship of embryo chromosomal aneuploidy with development to blastocyst stage.

Materials and Methods: A total of 369 biopsied embryos from 36 preimplantation genetic diagnosis (PGD) cycles of 26 patients were analyzed by the fluorescence in situ hybridization (FISH) for chromosomes 13, 18, 21, X and Y. The embryo biopsy was performed on Day 3 and all embryos were cultured to Day 5 and Day 6 to evaluate embryo development. Embryo transfer was performed on Day 5.

Results:
Of 26 patients, 54% chose male embryos for transfer, 30.8% chose female embryos for transfer and 15.4% patients transferred one male and one female embryo ($p<0.05$). Of the 369 embryos analyzed, 60.7% (224/369) were chromosomally normal, 29.3% (108/369) were abnormal and 10% (37/369) were unable to be analyzed. 74.1% (166/224) of chromosomally normal embryos developed to the blastocyst stage, but only 26.8% (29/108) of abnormal embryos reached the blastocyst stage. After PGD and embryo transfer, the overall pregnancy rate was 65.6% (21/32), but when only high quality blastocyst embryos were counted, the pregnancy rate reached 87.5% (21/24).

Of 224 chromosomally normal embryos, 46.4% were male and 53.6% were female, and their blastocyst development rates were 77.0% and 72%, respectively.

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Of 108 chromosomally abnormal embryos, 28-36% of trisomy embryos in 13, 18 and 21 chromosomes could develop to blastocyst stage, but the lack or aneuploidy of the 18 chromosome would limit the embryo development although the embryos with lack or aneuploidy of No. 13 and 21 chromosomes still developed to blastocyst stage. The embryo lacking the X chromosome could not develop to blastocyst stage, but the lack of Y chromosome seemed not to be a significant factor for embryo development to the blastocyst stage. X0 or XXY embryos could develop to blastocyst, but additional X or Y chromosome embryos such as XXX, XXYY, could not develop to blastocyst.

Conclusion: Based on these results, we concluded that aneuploidy of early embryos may have a significant effect on embryo development. Transferring chromosomally normal high grade blastocyst stage embryos results in a high rate of pregnancy.

Embryo Transfer On Day 3 Negatively Affects Blastocyst Conversion Of Surplus Embryos And May Influence Patient's Outcome When Compared With Day 5 Embryo Transfer

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Introduction: Many IVF Programs are considering transitioning from day 3 (D3) embryo transfer to day 5 (D5). For this purpose some centers may decide initially not to freeze surplus embryos on D3, initiating extended culture with the intent of producing blastocysts. D5 and D6 blastocysts of acceptable quality are then cryopreserved for future transfer. Others define systematically in advance which patients will receive some embryos on D3 and which ones will receive extended culture without D3 transfer mainly based on the number of fertilized (2PN) oocytes. Since January 2011 our program has transitioned from Day 2/3 transfer and freezing to blastocyst culture with no transfer or freezing on D3 on select patients.

Objective: This study attempts to evaluate the impact of D3 embryo transfer on blastocyst conversion and patient outcome using a novel drawer type trigas incubator.

Design: Retrospective analysis of ART cycles from patients receiving extended culture with and without D3 embryo transfer during 2011 and Q1 2012.

Materials and Methods: Fifty-seven patients undergoing ART (IVF/ICSI) were included in the analysis. Patients were grouped by embryo transfer day (D3: 24 or D5: 33). Conventional downregulation with GnRH agonist and GnRH antagonist LH suppression was used. Gonadotropin was used together with hCG triggering as part of COH protocol. LP support with P4 was also administered. The culture media system used included the following: Quinn's Advantage Medium w/HEPES plus 5mg/ml HSA (Sage IVF, Trumbull, CT), P1 (Irvine Scientific, Santa Ana, CA), Quinn's Advantage Protein Plus Fertilization Medium (Sage), Quinn's Advantage Protein Plus Cleavage Medium (Sage) and Quinn's Advantage Protein Plus Blastocyst Medium (Sage). Hyaluronidase and PVP used was also from Sage. Two IVF Cube (Astec, Fukuoka, Japan) incubators were exclusively used for these patients. Each incubator includes four drawers with independent control of temperature, CO_2 and O_2. Culture was conducted at 6% CO_2, 5% CO_2 and 37.0°C. The IVF Cube concept includes a contact incubator with a very small culture chamber. Recovery time after opening the culture chambers is three minutes. One or two patients per drawer were cultured and oocytes/embryos were examined and media changed on D1 and D3, minimizing embryo exposure to ambient conditions. Blastocyst conversion rate was obtained dividing the number of blastocysts obtained on D5-6 over the number of 2PN fertilized oocytes.

Results: There was no difference in the age of the patients between D3 (34.7±4.2) range 25-43 and D5 (34.2±4.1) range 28-43. The total oocytes recovered per group were 461 (D3) and 493 (D5) with a mean of 14.0±5.1 and 20.5±6.8 respectively ($p<0.0001$). Fertilization rate (IVF & ICSI) was 62% (D3) and 68% (D5). The overall blastocyst conversion rate was 51% (317/622) and it was 64.3% for D5 and 47.4% for D3 ($p<0.05$). Clinical pregnancy rate for D3 patients was 51.5% (17/33) and 70.8% for D5 (17/24) (NS). Miscarriage rate was 6.1% and 12.5% (NS) respectively.

Conclusion: Blastocyst conversion rate was higher on patients receiving D5 transfer versus D3. Clinical pregnancy was higher for D5 group but didn't reach statistical significance. The number of fertilized oocytes and embryos obtained influenced the decision for D3 or D5

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transfer so the blastocyst conversion rate could have been influenced by that decision alone. In any case this small retrospective study could help those centers transitioning from D3 to D5 to extend the culture without the need of a D3 transfer, minimizing the unlikely event of having no blastocyst available for transfer on D5.

Support: None.

Establishment Of An IUI Program For HIV-Discordant Couples In The United States

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Presentation of HIV-discordant (HIV-D) couples (male positive), unable to afford IVF, caused us to consider developing an HIV-D intrauterine insemination (IUI) program. An extensive literature search convinced us that this service should be provided because: 1) CDC guidelines recommend consistent condom use, thus necessitating fertility services; 2) HIV is a chronic disease and ADA requires clinics to provide equal treatment (upheld by the Supreme Court in *Bragdon v Abbott*); and 3) assisted reproduction for HIV-D couples is preventive medicine. The risk of HIV transmission is low, about 7% per year of unprotected intercourse and 0.1% per coital act. Seroconversion is further decreased by HAART treatment, low viral load and lack of concomitant STI. "Sperm washing" for IUI, consisting of gradient separation and swim-up, is standard of care in some countries. More than 4000 IUIs have been reported worldwide without seroconversion of the women or vertical transmission to babies. No seroconversion has ever been reported for HIV-D IUI with sperm washing. Although common worldwide, there are no published data of IUI for HIV-D couples in the US. Our protocol was IRB-approved and patients provide informed consent. In order to minimize risk and ensure optimal fertility per IUI, inclusion criteria require: 1) complete, normal fertility work-up; 2) female $\leq$ 40 years old; 3) good semen quality with $\geq$ 10 million motile sperm on day of IUI; 4) male under the care of an infectious disease specialist, with viral load < 50,000 copies/mL and stable CD4 count > 250 cells/mL; 5) couple negative for infectious diseases (gonorrhea, chlamydia, syphilis, HBV, HCV, HIV for female); 6) female is free of vaginal lesions; and 7) couple attests to consistently practicing safe sex. Couples are limited to 6 cycles of IUI. HIV-D cases are scheduled 3 hours before daily procedures begin, allowing sanitization of the collection room before regular patients arrive. Semen is collected by masturbation and processed using Universal Precautions within a biosafety cabinet (BSC) dedicated to this project. Contact materials are disposable except dedicated Makler chambers. Sperm are separated from raw ejaculates by gradient separation followed by swim-up. Centrifugation is performed in buckets with screw-top lids sealed in the BSC. Isolated sperm are washed once and prepared for swim-up, then tubes are sealed tightly and disinfected with 70% ethanol before transfer to the incubator. After washing the swim-up sperm, an aliquot is analyzed by RT-PCR/ELISA with appropriate positive and negative controls. PCR requires 8 hours, during which the sperm are held in the dark at room temperature. If positive and negative controls are accurate and the sperm sample tests negative for HIV, the sample is used for IUI. After each insemination, the female is tested for HIV at 3, 6, and 12 months. If pregnancy occurs, she receives prenatal care from a physician experienced with prevention of vertical transmission. To date, we have enrolled 4 couples, performed 2 IUIs, cancelled one IUI due to PCR detection of HIV RNA in the prepared sperm sample and have one pregnancy with normal, late first trimester ultrasound.

Is There A Benefit To Performing Intracytoplasmic Sperm Injection On Late Maturing Oocytes?

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Objective:
To compare the developmental potential of oocytes that develop a polar body after initiation of ICSI (Late MII) with those that have a polar body at the start of the ICSI procedure (MII).

Design:
A retrospective study of 164 autologous IVF cycles performed 1/1/07 through 2/22/11 in which ICSI was performed on 1617 MII oocytes and 238 Late MII oocytes.

Materials and Methods:
Intracytoplasmic Sperm Injection (ICSI) was performed 4-5 hours post-retrieval on oocytes that had matured to

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the MII stage. After all MII oocytes were injected with sperm, the maturity of the remainder of the ooctyes was re-evaluated. Any oocytes that developed a polar body since the last observation (Late MII) were also injected with sperm. If <5 oocytes were injected, the observation for appearance of Late MII oocytes was continued until 5-6 hours post-retrieval. Embryos were cultured individually in Sage media in 5% O_2. Fertilization rate, day 3 embryo quality and blastocyst quality (Day 5 transfers only) for MII and Late MII oocytes were compared.

Determination of transfer day:
Transfer was performed on day 3 if <35 years and fewer than 4 Top quality embryos; 35-40 years with fewer than 6 Top quality embryos; or patients >40 years regardless of embryo quality. All other patients were transferred on day 5.

Definitions of embryo quality:
Good Quality Day 3 Embryo: An embryo with the following characteristics at the indicated time points post-insemination: 25 hours: Between the 2 pronuclear stage and 2 cell stage, even cells, no multinucleate blastomeres; 42-44 hours: 2-5 cells, even cell divisions, no multinucleate blastomeres; 66-68 hours: 7-8 cells with <20% fragmentation or 6-9 cells with <15% fragmentation, ≥2 more cells than on day 2, even cell divisions, no multinucleate blastomeres.

Good Quality Blastocyst: A blastocyst with the following characteristics on either day 5 or day 6: Expanded blastocoel cavity, organized and compacted inner cell mass, medium to large number of cohesive cells in the trophectoderm.

Statistical analysis: Data were analyzed using Pearson Chi Square.

Results:

Oocyte Type	% Fertilization	% Good Quality Day 3 Embryos	% Good Quality Blastocysts
MII	74.6% (1207/1617)	57.3% (693/1209)	15.6% (80/512)
Late MII	53.4% (127/238)	52.0% (66/127)	13.5% (5/27)
P	0.000	0.247	0.064

Conclusion:
Late MII oocytes fertilize at a fairly good rate, even though it is significantly lower than that of MII oocytes. The Late MII oocytes that fertilize develop into good quality embryos/blastocysts at a rate equivalent to that of MII oocytes. Insemination of Late MII oocytes is therefore of benefit to patients.

MicroSecure Vitrification (µS-VTF): Evaluation of Thaw Intervals for Human Blastocysts

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Objective: MicroSecure vitrified (µS-VTF) blastocysts (BLs) were thawed to determine whether the ambient temperature thawing interval could be reduced safely without compromising viability and allow further study on VTF-oocyte thawing intervals. Cryophysical models suggest a delicate balance between cellular rehydration/equilibration and membrane plasticity. BLs vitrified in non-DMSO solutions were thawed and evaluated in three test time groups for survival and viability of shorter sucrose dilution intervals. We aimed to determine that a 5 min dilution at each of 5 dilution steps, as indicated by the manufacturer, is excessive. We proposed that the dilution intervals could be reduced to 3 min without adversely altering cellular viability.

Methods: In Phase I, 55 discard µS-VTF BLs were randomly separated into three treatment group dilution intervals (n=14/trt): 1min, 3min, and 5min (positive control). All embryos used were research consented. BLs were diluted stepwise from 1.0M sucrose (T1) to isotonic LG-H (HEPES buffered T5) in five intervals. Note, in the 1min treatment group BLs were exposed to T1 solution for 3 min before applying the 1min intervals (T2-T5). In addition, a negative control group (n=13) was added to evaluate BLs in T1 solution for 3 min before direct placement into LG-H. After dilution, all VTF BLs were incubated with 5% CO_2 at 37°C and cultured using LG medium + 7.5%SS in microdrops under oil for 24 hrs. The BLs were assessed for continued development (BL expansion/hatching=survival). In the 1st quarter of 2012, Phase II of this study was implemented to evaluate the effectiveness of 3 min µS-VTF BL dilution intervals on FET cycle outcomes (n=44, all age groups). We contrasted these preliminary outcomes to our µS-VTF FET cycles in 2010-2011 (n=206).

Results: No differences in post-dilution survival of VTF BLs were observed in all treatment groups. Furthermore, survival in our negative control group was not different.

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Dilution Interval	5min	3min	1min	Negative Control
# Thawed	14	14	14	13
# Survived (%)	13 (93%)	13 (93%)	13 (93%)	12 (92%)

In the clinical application of the 3 min dilution treatment, 80 of 81 BLs survived (98.8%), and 32 of 44 (72.7%) developed a clinical pregnancy. The outcomes compare favorably to 2010-11 outcomes for BL survival (398/420, 95%) and clinical pregnancy rates (n=134, 65%).

Conclusion: VTF BLs were more resilient to osmotic changes than theoretical modeling would predict. 1M sucrose effectively and safely dehydrates BLs, eliminating the potentially toxic intracellular cryoprotectants, with normal membrane fluidity post-VTF. Most notable, the negative control demonstrates that an initial 3 min 1 M sucrose exposure resulted with comparable survival. Based on our results, the 3 min/ 5 dilution step protocol has been successfully implemented into our clinical practice with continued consistency in survival and pregnancy. Additional viability studies are needed before more extreme thawing practices (e.g., 1 min dilutions or single step only thaw) are adopted for clinical application. Considering the membrane resiliency of VTF BLs during rehydration without lysis, we are hopeful that these findings can be applied to human oocytes. Ongoing investigations are aimed at optimizing our aseptic vitrification system for oocytes using the non-commercial, FDA compliant µS-VTF system. Overall, microSecure vitrification (Schiewe, JCE, 2010) offers unparalleled facets of quality control (e.g., labeling, security, technical ease, repeatability, and storage) which should be seriously considered in the clinical application of vitrification.

Disclosure: The authors have no commercial interests in the µS-VTF product.

Potential For Empowering And Broadening The Application Of SART Embryo Grading System

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Objective: Morphological assessment has been the method of embryo selection since the application of IVF for infertility treatment. The necessity of developing a unifying embryo grading system acceptable to all has since been felt. Recently SART took such an initiative so that embryo grading is done using a uniform grading system in reporting embryo data to SART registry. SART elected three growth phases, cleavage, morula and blastocyst, to be evaluated by such grading. The objective of the present study was to see if the SART grading can be strengthening further, and be applied to other growth phases of the embryo.

Study Design: Possibility of integration of more morphological determinants into the SART grading system, and its application to all growth phases are explored.

Materials and Methods: The grading system developed by SART task force and the preliminary outcome of the collection of the information based on such grading were assessed. Proficiency test (PT) data on SART grading system were analyzed. Published literatures on different embryo grading models were reviewed. Morphological determinants used in previous grading systems but not in SART system were attempted to incorporate in the SART three point (good, fair, poor) grading system, and the possibility of its uses to all growth phases is assessed.

Results: The SART three point grading (good, fair, poor) utilized an independent set of three parameters for cleavage and blastocyst, and two parameters for morula. Grading morula is an unpopular event. Proficiency testing did not include day 4 morula; instead it targeted day 1 zygote, day 3 cleavage and day 5 blastocyst. The post insemination timing of grading embryos was 17-18 hrs, 42-44 hrs, 64-68 hrs, 88-90 hrs, 115-117 hrs and 140-142 hrs for day 1, day 2, day 3, day 4, day 5 and day 6 of growth, respectively. A set of four morphological parameters in each growth phase have been proposed in which all relevant morphological determinants that were utilized so far in the previously reported embryo grading models can be accommodated. Further, these growth phase specific morphological parameters can be integrated into the SART three point grading system for evaluating any developmental stage starting from day 0 to day 6 of in vitro development.

Conclusion: The SART embryo grading can be made powerful by incorporating more relevant morphological determinants into it without compromising its simplicity and uniqueness. Secondly, the SART system is applicable in any preferable day of embryo assessment.

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Spermatozoa With Normal And Abnormal Head Morphology Exhibit Different Staining Patterns

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Objective: Performing semen analysis is a routine task in an andrology laboratory and the study of spermatozoa's morphology is an integral part of semen analysis. Different types of stain are used in assessing sperm morphology. However, the objective is the same — to have a better view of the cellular components so that deformity of any kind can be identified. In this study we explored the staining pattern of spermatozoa possessing normal and abnormal head when stained with STAT III andrology stain.

Study design: Intensity and pattern of stain exhibited by sperm with abnormal head was compared with that of normal head.

Materials and Methods: Thin semen smears of apparently normal semen were prepared on clean glass slide and air dried for 30 minutes. The slides were stained with STAT III andrology stain following manufacturer's suggested protocol (Mid-Atlantic Diagnostics, Mt. Laurel, NJ). Spermatozoa (n=500) were evaluated under oil immersion with equal representation of normal and abnormal head. Each spermatozoon was scored for head morphology (normal or abnormal) followed by the assessment of color intensity and pattern (A, B or C). The criteria of sperm head normality was that of the WHO manual 3rd edition. The color exhibited by the sperm head was branded as A) light yellow-reddish (dominance of eosin Y), B) dark blue (dominance of azure), or C) equal dominance of both. Pearson's chi-square test was used to check the equality of normal and abnormal sperm for each of the stain pattern.

Results: The color intensity in all three stain categories (A, B and C) was higher in abnormal head compared to normal head. Normal head exhibited a color pattern in the order of A > C > B while the pattern exhibited by the abnormal head was B > C > A. The 43% and 22% sperm with normal and abnormal head, respectively exhibited color A (chi-square 25.96, $p < 0.0001$) while 19% and 52% sperm with normal and abnormal head, respectively demonstrated color B (chi-square 60.46, $p < 0.0001$). The color C was exhibited by 38% and 27% sperm with normal and abnormal head, respectively (chi-square 7.03, $p < 0.0080$).

Conclusion: There were augmented color intensity, and different color pattern of sperm having abnormal head morphology compared to sperm with normal intact morphology. This color difference probably indicates that morphological deformities increase the exposure of the cellular components to stain ingredients.

Theoretical Investigation Of Slow Freezing Protocols For Human Oocytes In The Presence Of 1,2-Propanediol

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Objective: Cryopreservation protocols can be developed by theoretically examining biophysical events during the process.

Design: Theoretical calculation and simulation.

Materials and Methods: A theoretical model makes it possible to investigate the biophysical characteristics of human oocytes undergoing a slow freezing procedure. To develop protocols, the goal is to determine the best selection of the initial CPA concentration ([CPA]), cooling rate (B) and the plunging temperature (Tp). This theoretical model was applied to human oocytes using 1,2-propanediol as the CPA. An initial range of concentrations from 0 to 4 M, and a range of cooling rates from 0 to 1.5°C/min were evaluated theoretically to select [CPA] and B that would allow the intracellular solute concentration([S]) to reach the critical concentration ([CPA]c). Using Mazur's IIF model, conditions that could result in IIF were eliminated. The associated plunging temperatures were then calculated.

Results: When the results were plotted on the plane of [CPA] and B, three regions emerged with their own characteristics. The combinations of [CPA] and B residing in Region I and II would result in IIF damages, and hence were rejected. In Region III, the combinations would allow the [S] to reach the [CPA]c, and no IIF was predicted. Roughly, there is a minimum [CPA] boundary of 1.25 M

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and there is also a maximum B boundary of 0.4°C/min. The plunging temperatures for these combinations were determined and ranged from -34°C to -38°C.

Conclusions: A theoretical model can be used for the development of cryopreservation protocols by designing specific cooling profiles and selecting conditions to optimize slow freezing. Compared to the published protocol (-0.3°C/min cooling rate, plunging at -30°C), the current study suggests the plunging temperature should be approximately -38°C. A more conservative protocol (1.5 M, -0.2°C/min , at -36°C) is recommended and plans are underway to test this protocol.

Support: Laura W. Bush Institute & UMC for Women's Health.

Validation Of A Line Blot Assay For Diagnosis Of Lyme Disease

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Background: Lyme disease, the most common vector-borne disease in the United States, is a tick-born multisystem disease caused by *Borrelia burgdorferi* infection. The Centers for Disease Control and Prevention currently recommends a two-tier serologic approach for laboratory diagnosis, comprised of an initial enzyme immunoassay screening supplemented by IgG and IgM immunoblot for any screen-positive or equivocal samples. Western blot assay is the most widely used method for the second-tier test. This method utilizes a whole-cell lysate of *B. burgdorferi* as antigen, which is separated by SDS/PAGE and then transferred to a nitrocellulose membrane. However, the whole-cell lysate contains both specific and nonspecific antigens resulting in co-presence of diagnostic and non-diagnostic bands, which is inconvenient and error-prone for result interpretation. Moreover, it is difficult to maintain consistency in the quantity of each antigen on the strip which hampers standardization of the test. Recently an FDA-cleared line blot assay became commercially available. This line blot is generated by imprinting isolated and purified whole cell lysate antigens at consistent concentrations and defined locations, onto a nitrocellulose membrane. We performed a validation study on the IgM and IgG line blot assay by comparing it with a widely used Western blot assay.

Method: The line blot kits (ViraStripe®, Viramed/Germany) and Western blot kits (MarBlot, Trinity Biotech/USA) were compared by testing a panel of 102 human serum samples. Results were interpreted according to the CDC assessment criteria by at least five laboratory specialists. Cross reactivity was evaluated by testing 8 syphilis and 8 anti-nuclear antibody positive specimens. Analytical sensitivity was compared by testing serial dilutions of a strongly positive specimen.

Results: The positive and negative agreement between the ViraStripe and MarBlot IgM assay was 82.4% and 95.5% respectively. MarBlot diagnosed more positives (34 samples) than the ViraStripe (31 samples). Fifty percent of the positive specimens identified by both assays showed disagreement in significant bands. The band which exhibited highest discordance in interpretation is band 39. Among the negative specimens, band 41 was more frequently positive by ViraStripe (11% in ViraStripe vs. 5% in MarBlot) while band 23 was more likely to become positive by MarBlot (13% vs. 6%). The two IgG assays showed 87.5% positive agreement and 100% negative agreement. Thirty two percent of specimens positive by both assays were found to be discordant in more than 5 of the 10 significant bands. The analytical sensitivity of the VirStripe IgM and MarBlot IgG assay were higher than their counter partner demonstrated by their capability of detecting more diluted samples. None of the assays showed cross reaction in specimens containing anti-nuclear antibody. One out of eight specimens was positive by both IgM immunoblot.

Conclusion: The Borrelia line blot IgM and IgG assay showed acceptable concordance to a widely used Western blot assay, and is clinically useful in diagnosis of Lyme disease. However, some discordance in IgM results and remarkable disagreement in significant bands should not be neglected. Further improvement and standardization of the Lyme disease second-tier tests is necessary.

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Vitrification Of Blastocysts In CBS Straws: Comparison Of Two Methods

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Objective: To decide if a difference exists between two methods of blastocyst vitrification in CBS Straws.

Design: A retrospective study comparing viability, implantation and ongoing pregnancy rates of contrasting vitrification methods. Data from 82 FET cycles (69 patients) performed between September 24, 2009, and February 22, 2011, were analyzed.

Materials and Methods: Embryos were cultured in Sage media in 5% O_2. Blastocysts of freezable quality were vitrified on Day 5 or Day 6. Freezable quality is defined as a blastocyst with an expanded blastocoel cavity, a well organized and compacted ICM, and a trophectoderm comprised of a medium to large number of cohesive cells. All blastocysts were vitrified and thawed using S^3 Vitrification and Thaw media with equivalent exposure times for both procedures.

Direct Straw Method: A single blastocyst was pulled directly into a 0.3mL CBS High Security Embryo Straw (CBS straw) in 200uL medium followed by an air pocket. The straw was immediately heat sealed on both ends and placed in vapors in a Planar Controlled Rate Freezer at -100ºC for 5 minutes before plunging into LN_2. To thaw blastocysts, the straw was removed from LN_2 and exposed to room temperature air for ten seconds followed by ten seconds in a 30ºC water bath. The blastocyst was then expelled into a 400uL pool of the first thaw dilution and allowed to acclimate for 30 seconds before being transferred to a fresh drop of the same medium. The blastocyst was then moved through subsequent dilutions.

MicroSecure Vitrification (µS-VTF; Schiewe): Medium containing the blastocyst was drawn into a shortened Stripper Tip. The Stripper Tip was then immediately placed into the CBS straw, heat sealed at both ends and plunged directly into LN_2. To thaw these blastocysts, the CBS straw was removed from LN_2, the end immediately cut and the Stripper Tip dropped into a 60mm culture dish containing 13mL of 37ºC 1M Sucrose/HTF-HEPES so that the end containing the blastocyst was submerged. The blastocyst was then expelled into the first thaw dilution and moved through all remaining dilutions.

Statistics:
Poisson Distribution: Viability and Implantation Rate
Logistic Regression: Ongoing Pregnancy rate

Results:

Method	Viability	Implantation Rate (Ongoing Heartbeats/# Blastocysts Transferred)	Ongoing Pregnancy/Transfer
Direct Straw Method	56.3% (80/142)*	32.9% (27/82)	42.8% (21/49)
µS-VTF	91.7% (44/48)*	36.4% (16/44)	50.0% (12/24)

*P=<0.001

- The viability of blastocysts vitrified by the µS-VTF method is significantly higher than the viability of blastocysts vitrified by the Direct Straw Method.
- For blastocysts that survive the thaw, the implantation rate and ongoing pregnancy rate are the same for the two vitrification methods.

Conclusion:
The µS-VTF vitrification method results in better outcomes than the Direct Straw Method because of the significant improvement of blastocyst viability.

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What Is The Perceived "Gold Standard" For Sperm Counting Chambers? A Survey

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Objective: In the andrology field there is a great demand for accurate tools to help men that have fertility problems. Semen analysis has been a crucial tool for evaluation of the male partner in many situations which looks at the quantity of quality of the sperm. These situations include: post-vasectomy analysis, infertility evaluations, as well as sperm banking. The method(s) of semen analysis

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are almost universally the same, except for the counting devices used for counting. Many publications (e.g. WHO Manual) default to the hemocytometer as the "Gold Standard" for such counting chambers. However, since the hemocytometer was originally designed for use in counting other cell types (e.g. blood cells) and FDA approved sperm counting chambers are available, we were interested to learn which sperm counting chamber is most commonly used.

Design: Analysis of CAP Proficiency Testing (PT) data (2009-2011) and EmbryoMail survey.

Materials and Methods:
1) In the CAP PT survey, close to 12,000 results were reviewed on what device was reported. The different devices reported included Microcell, Hemocytometer (reusable), Cell-VU, and Makler (reusable).
2) In the Embryo-Mail Survey, Microcell, Hemocytometer, Cell-VU, Makler, and Leja were given as choices.

Results:

Counting Chamber	CAP PT (2009-2011)	EmbryoMail
Hemocytometer	**7975(67%)**	8(18%)
Makler	1360(11%)	**20(45%)**
Cell-VU	819(7%)	3(7%)
MicroCell	517(4%)	9(20%)
Other	1278(11%)	4(9%)
Total	11949	44

Conclusions: From the two different surveys, one thing that can be concluded is that the reusable counting chambers have a much larger usage than the disposable chambers. One primary reason is due to cost and not due to performance purposes, as some respondents noted. Some positive attributes from reusable chambers are (1) helps reduce cost by not having to constantly buy new slides, and (2) permits data comparison with a greater number of labs. Some negative attributes of the reusable chambers include (1) time to clean the chambers between uses, (2) usage and wear on the devices that may give less accurate results over time and (3) dilution/calculation errors. Looking at pros and cons without considering price, the disposable chambers have a valid argument. Since they are disposable they are always brand new for use making the results unaffected by usage and wear, and allowing a minimal chance of cross contamination. The biggest negative for disposable chambers would be the cost and also the low percentage of labs using these chambers for data comparisons. While it is still difficult to state that the hemocytometer is the golden standard based on performance, it certainly is a favorite from users as reported in the CAP PT, the Makler was the leading favorite from the EmbryoMail survey. The question now is, does the hemocytometer actually perform the best? Do reusable chambers outperform the other chambers tested? And lastly, if it is proven that the disposable chambers do perform better, and then it comes down to money versus performance, what will labs decide to do, keep cost down and stay with the reusable chambers, or absorb the extra cost and have a more accurate semen analysis? This could be a deciding factor on what labs will decide to use for semen analysis.