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Oral Communications

CFAS Exchange Lecture

Noriko Kobayashi's research was selected by the ART Laboratory Special Interest Group of the Canadian Fertility and Andrology Society. Her outstanding research was recognized and awarded the 'Alpha Exchange Award' provided through an unrestricted educational grant from Organon, Canada at the 53rd CFAS Annual Meeting, September 2007 held in Halifax, Nova Scotia.

Regulation of rat preantral follicular development by FSH and thyroid hormone: involvement of growth differentiation factor-9

Kobayashi N¹, Orisaka M², Kotsuji F², Leader A¹, Sakuragi N³, Tsang BK¹

¹Department of Obstetrics and Gynecology and Cellular and Molecular Medicine, University of Ottawa; Ottawa Health Research Institute, Ottawa, Ontario, Canada; ²Department of Obstetrics and Gynecology, University of Fukui, Fukui, Japan; ³Department of Gynecology, Hokkaido University Graduate School of Medicine and School of Medicine, Sapporo, Japan

Objective: Although it is well established that hypo- and hyperthyroidism is associated with suppressed ovarian follicular growth and infertility, the mechanism(s) involved is not known. Growth differentiation factor-9 (GDF-9) increases rat ovarian follicular growth *in vitro*. While FSH also promotes growth of cultured follicles, this response is markedly enhanced by the presence of GDF-9 or tri-iodothyronine (T3). While GDF-9 is required for FSH receptor (FSHR) expression in preantral follicles, whether this response is required for the FSH-T3 interaction during preantral growth is unclear. The objective in the present study is to assess the role of GDF-9 in the increased responsiveness of the preantral follicles to FSH and T3 stimulation and the cellular mechanism involved.

Materials/Methods: Preantral follicles (diameter: 150–180 μm) isolated from ovaries of 14-day old rats were cultured in serum-free medium for various durations (0–60 h) and concentrations of FSH (0–100 ng/ml) or T3 (0–10–8 mol/l). FSHR mRNA abundance and 18S rRNA concentrations were determined by quantitative real-time PCR and FSHR mRNA concentration was expressed as a ratio to 18S rRNA values. GDF-9 morpholino antisense oligos (GDF-9 MO, 10 $\mu\text{mol/l}$) or its control morpholino (CTL MO, 10 $\mu\text{mol/l}$) were injected into the oocyte and the follicles were cultured for 4 days with or without FSH and T3. Follicular diameter was determined during the culture period.

Results: FSH and T3 significantly increased preantral follicle growth during a 4-day culture period, a response completely suppressed by intra-oocyte injection of GDF-9 morpholino ($P < 0.001$). Addition of GDF-9 (100 ng/ml) to the morpholino-treated follicles significantly prevented the decrease in preantral follicular growth ($P < 0.01$). T3 significantly increased the level of FSHR mRNA in the presence but not absence of FSH at 36 h of culture ($P < 0.01$). Maximal responses were observed at 10 ng/ml FSH and 10^{-9} mol/l T3.

Conclusion: The findings suggest that FSH and T3 interact in promoting preantral follicle growth, and this regulation is mediated through GDF-9-dependent FSHR mRNA expression. This study offers new insight into the molecular and cellular

mechanism of impaired follicular growth during thyroid hormone dysregulation.

This study was supported by CIHR-IHDCYH Program on Oocyte Health and STIRRHS).

Cryopreservation

Cryobanking for oocyte donation programmes by the use of the Cryotop vitrification method

Cobo A, Budak TP, Meseguer M, Perez S, Zulategu J, Albert C, De Los Santos MJ, Remohi J

Instituto Valenciano De Infertilidad, University of Valencia

Objective: Cryobanking for oocytes in an oocyte donation program will considerably boost the programme in an effective and safe manner, since no synchronization between donors and recipients is required and oocytes are kept in a quarantine period similar to semen banks. In spite of the advantages provided by freezing oocytes, cryopreservation techniques traditionally have failed to produce convincing results to allow its incorporation into routine clinical practice. Recent advances in oocyte and embryo cryopreservation techniques have been achieved with vitrification, especially with the Cryotop method. The objective of this study is to report the clinical results obtained after donation of vitrified oocytes with the Cryotop method.

Materials/Methods: Forty-seven donors and 57 oocyte recipients were included in this study. A total of 693 MII oocytes were collected after ovarian stimulation using the long protocol down-regulation plus gonadotrophin administration. Vitrification was carried out with the Cryotop method. Oocytes were donated to a recipient once the endometrial preparation was adequate to proceed with the donation and embryo transfer procedure.

Results: Of the 693 cryopreserved oocytes, 666 (96.1%) survived. A total of 487 (73.1%) oocytes were normally fertilized. 57 embryo transfer procedures were performed. The mean number of embryos transferred was 1.99 ± 3.7 . The pregnancy rate was 63.2% and the implantation rate was 38.5%. The multiple pregnancy rate was 30.5%. The miscarriage rate was 16.6% and the biochemical pregnancy rate was 13.9%.

Conclusion: Results obtained after donation of vitrified oocytes using the Cryotop method, demonstrates that this technology represents a great alternative for the creation of cryobanking for oocyte donation programs. In this study, excellent cryosurvival rates as well as clinical outcome parameters were obtained similar to those in our current oocyte donation program using fresh oocytes.

Embryo culture

Reducing the time of sperm-oocyte exposure does not improve conventional IVF cycle outcome

Grassa LH¹, Ramírez JM¹, Mínguez Royo Y¹, García-Velasco JA²

¹IVI Madrid, Spain; ²Rey Juan Carlos University, Madrid, Spain

Objective: In recent years, a lot of studies have been published on the effects of reducing the exposure of the oocyte to the

spermatozoa in IVF cycles. Some of them conclude that this co-incubation time reduction improves cycle outcome, perhaps because of the decreasing of oxidative stress. The aim of this study was to examine the effect of a reduction in the time of co-incubation of gametes, in terms of fertilization and triploidy rate, embryo quality, pregnancy and implantation rates.

Materials/Methods: This prospective randomized study includes 75 patients who underwent a conventional IVF cycle in our centre between January and July 2007. Each patient was assigned to one of these groups: (i) study group (35 patients, 340 oocytes): oocytes of these patients were co-incubated for only 1 h with spermatozoa, and then the oocytes were allocated in fresh HTF drops without spermatozoa, and were incubated overnight; (ii) control group (40 patients, 262 oocytes): standard overnight co-incubation (16–19 h). In both groups, oocytes were incubated in HTF with a final sperm concentration of 200,000/ml. After assessment of fertilization (16–19 h post-insemination), the zygotes were transferred to fresh HTF drops and cultured until embryo transfer in day 2 or 3. Statistical analysis was carried out by t-test and chi-squared test, and considered significant if $P < 0.05$.

Results: The results are shown in **Table 1**.

Conclusion: (i) The higher exposure time, the higher fertilization rate; (ii) triploidy rate does not increase with a higher exposure time; (iii) we observed higher pregnancy and implantation rates in the overnight co-incubation group; (iv) embryo quality is similar in both groups, but number of blastomeres on day 3 is significantly higher in the study group, in spite of that this group achieved better cycle's outcome. We could not conclude that the oocyte suffers oxidative stress in the overnight incubation situation, because all our parameters are similar or better in that group comparing with the study group.

Table 1.

	<i>1-h co- incubation</i>	<i>Overnight co- incubation</i>	<i>P-value</i>
No. oocytes	340	262	
Mean oocyte age	35.05	34.55	NS
Mean oocytes/patient	9.65	6.57	0.0132
Fertilization rate %	51.91	64.85	0.0250
Triploidy rate	10	10.30	NS
Mean day-2 blastomeres	4	3.97	NS
Mean day-3 blastomeres	7.66	6.93	0.0003
Top quality embryos %	30.77	29.19	NS
Mean transferred embryos	2.0	1.57	0.026
Frozen embryos %	30.56	25.53	NS
Pregnancy rate %	41.18	58.82	NS
Implantation rate %	26.47	36.76	NS

Impact of specific embryo characteristics on ongoing implantation in unselected embryos derived from modified natural cycle IVF

Arts EGJM¹, van Echten-Arends J¹, Vogel NEA¹, Hoek A¹, Simons AHM¹, MJ Heineman², Pelinck MJ¹

¹Section of Reproductive Medicine, Department of Obstetrics and Gynaecology, University Medical Centre, Groningen, The Netherlands; ²Department of Obstetrics and Gynaecology, Academic Medical Centre, Amsterdam, The Netherlands

Objective: In IVF, knowledge of implantation according to embryo characteristics is essential but difficult to study, due to selection of embryos for transfer. In contrast, in modified natural cycle IVF (MNC), usually one embryo is available, making it possible to study the implantation potential of unselected embryos. In the present study, a series of 449 single embryos derived from MNC-IVF was studied and the impact of specific embryo characteristics on implantation was calculated.

Materials/Methods: All patients were younger than 38 years with conventional IVF indication. A GnRH antagonist and FSH were given only in the late follicular phase of the natural cycle. Conventional IVF was applied according to standard procedures. Oocyte/embryo morphology was assessed at 19, 43 and 67 h after insemination. All embryos were transferred on day 3 without further selection, except those that showed ≥ 3 pronuclei, $\geq 50\%$ fragmentation or no cleavage. Logistic regression analysis was applied to determine the influence of embryo characteristics on ongoing implantation. Embryo characteristics tested were: PN score (2PN versus other), blastomere number on day 2 (2 or 4 versus 3 or 5), blastomere number on day 3 (8 versus 3–7), amount of fragmentation on day 3 ($\leq 10\%$ versus 10–40%), presence of multinucleation and cleavage rate (number of blastomeres on day 3 divided by number of blastomeres on day 2).

Results: Between January 2001 and September 2005, 449 single embryo transfers were performed, resulting in 99 ongoing pregnancies (ongoing implantation rate 22.0%). The best implantation was found in embryos with 4 and 8 cells on day 2 and 3 respectively, $\leq 10\%$ fragmentation and absence of multinucleation (43.4% ongoing implantation). Embryos deviating from this optimal development pattern by lower numbers of blastomeres show less impaired implantation than those with higher numbers. Best cleavage rates were found to be 2 and 2–3 (30.1 and 28.9% ongoing implantation respectively). PN score had no significant impact on implantation. Odds ratios found with multivariate analysis were 2.06 and 2.10 for blastomere numbers on day 2 and 3, 1.48 for fragmentation on day 3 and 1.56 for presence of multinucleation (P -values 0.08, 0.02, 0.19 and 0.28). For cleavage rate (2–3 versus 2, 1–2 versus 2 and ≥ 3 versus 2 respectively) odds ratios of 1.89, 0.85 and 0.31 were found (P -values 0.16, 0.61 and 0.07).

Conclusion: The results of this study suggest that in currently used embryo scoring systems, the implantation potential of embryos with lower numbers of blastomeres is underestimated. The number of blastomeres appears to have more impact on implantation than the amount of fragmentation or presence of multinucleation. Findings from this study can be used to improve selection of the best embryo for transfer.

Obtaining competent oocytes

Level of granulocyte-colony-stimulating factor reflect a successful stimulation and is a predictor of IVF outcome

Salmassi A, Jonat W, Mettler L, Schmutzler AG

Department of OB/GYN, Campus Kiel, University Hospitals Schleswig-Holstein, Germany

Objective: Granulocyte-colony-stimulating factor (G-CSF) is produced by haemopoietic cells and several non-haemopoietic cell, such as human reproductive tissue cells

Materials/Methods: Granulosa cells (GC) were separated from follicular fluid (FF) by the Percoll technique ($n = 21$). The protein expression of G-CSF and its receptor in GC were detected by immunocytochemistry and by RT-PCR technique. The concentrations of G-CSF in serum and in FF were measured in 82 IVF/ICSI patients (group 1) on the day of follicular puncture (FP) by ELISA. In group 2 ($n = 23$) G-CSF levels were measured in serum in all cycle phases until gestation.

Results: RT-PCR and immunocytochemical results demonstrated that human follicular GCs express G-CSF and its receptor. In group 1 the median G-CSF level in FF was significantly higher than that in serum ($P < 0.05$). Patients with poor response showed the lowest G-CSF concentrations in serum (40 pg/ml) and no pregnancy. Patients with moderate response displayed higher G-CSF concentrations in serum (59 pg/ml) and a pregnancy of rate 24.5%. Patients with good response showed the highest G-CSF concentration in serum (72 pg/ml) and a pregnancy rate of 33.5%. ($c2, P < 0.001$). In group 2, the levels of G-CSF on the day of HCG injection were significantly higher than during the early follicular (EF) and mid-follicular phase ($P = 0.01$). It decreased through FP to the day of embryo transfer (ET, $P = 0.001$). In the early luteal phase from the day of ET to the time of implantation ($P = 0.05$) and from the day of confirmation of pregnancy to gestation ($P = 0.005$), the G-CSF levels of those patients who became pregnant ($n = 11$) increased continuously and reached a maximum. If implantation failed ($n = 12$), G-CSF decreased to the level of the EF phase

Conclusion: The data suggest that G-CSF may play a role in proliferation and differentiation of granulosa cells and may be involved in follicle development and ovulation via an autocrine/paracrine mechanism within the follicular microenvironment. It could also be a predictor of embryo implantation for IVF outcome

Spindle position; a comparison of in-vitro and in-vivo matured human oocytes. A prospective comparative study in humans

Munk M, Novella-Maestre E, Lindenberg FB, Lindenberg S Nordica Fertility Clinic, Section for Reproduction and ART

Objective: Incomplete cytoplasmic maturation of in-vitro matured (IVM) oocytes has been known to cause microtubule and filament alterations, which may results in abnormal pronuclear formation and later failed embryogenic development. The present study examined the influences of in-vitro maturation of human ova to the MFII stage in a standardized in-vitro maturation medium compared with in-vivo matured MFII stage ova prior to the ICSI procedure.

Materials/Methods: A total of 44 patients admitted for IVM and 44 patients admitted for IVF/ICSI were analysed using a PolScope for evaluating the presence and placement of the

spindle in relation to the polar bodies in all MFII oocytes produced after egg collection prior to ICSI or after 28 h in-vitro maturation following the IVM protocol.

Results: We found the same mean number of oocyte retrieved (IVM 4.9 oocytes versus ICSI 5.9 oocytes). The mean numbers of MFII oocytes after collection of IVF/ICSI oocytes were 5.1 versus 2.1 for the IVM oocytes after 28 h maturation ($P < 0, 01$), and finally a significantly higher rate of displacement of the spindle apparatus was found in the in-vivo-matured oocytes compared with the in-vitro-matured oocytes ($P > 0.05$).

Conclusion: These findings demonstrate a considerable loss of MFII not developing to the MFII in the IVM patients, but the MFII oocytes produced after IVM are normal and have a better spindle position than oocytes directly collected after in-vivo maturation during regular IVF/ICSI procedures. This indicates that IVM matured oocytes if they reach the MFII are normal, and the obvious lower implantation rate in IVM might be due to other factors such impaired endometrial development during the IVM procedure.

Studies on transcriptional profiling of granulosa cells from patients undergoing infertility treatment

Coskun S, Al-Alwan L, Awartani K, Al-Rejjal R, Al-Hassan S, Out H, Inan MS

King Faisal Specialist Hospital and Research Centre

Objective: In recent years, transcriptional profiling by microarray technology has been shown as a promising tool to understand molecular mechanism in various physiological and drug-induced biological processes. Ovarian stimulation protocols are employed to stimulate multiple follicular growths and human chorionic gonadotrophin (HCG) injections are administered to induce oocyte maturation and ovulation. In certain cases, the dose of HCG is adjusted to prevent ovarian hyperstimulation syndrome. On the other hand, while some patients have all matured oocytes retrieved, others yield high number of immature oocytes after similar ovarian stimulation protocols. The objectives of this study were to compare the transcriptional profiling of granulosa cells in patients who took different doses of HCG and patients with different oocyte maturation outcome after similar treatments.

Materials/Methods: RNA isolated from granulosa cells collected from patients undergoing infertility treatment was analysed with Affymetrix GeneChip microarrays which consist of more than 39,000 genes. In the first experiment, patients with polycystic ovarian syndrome were included. They were stimulated with HMG. HCG injection was either withheld or administered according to the degree of response to HMG. In the second experiment, gene expression profiles of granulosa cells from patients with all mature oocytes retrieved were compared with the patients with more than 25% germinal vesicle (GV) stage oocytes retrieved.

Results: Hierarchical clustering analysis based on whole gene expression revealed distinct groups of patients in both experiments. In patients where HCG injection was withheld were clustered into one group, whereas the treated patients had completely different gene expression profile. Over 6000 genes were differentially expressed in response to HCG injections including genes involved in cell cycle, insulin signalling, WNT signalling and TGF-beta signalling pathways. In the second experiment, clustering analysis based on the presence of GV oocytes was very distinct between two groups and over 1000 genes were differentially expressed. Pathway analysis from

ingenuity system showed that the VEGF signalling pathway and hypoxia signalling pathway were highly dysregulated.

Conclusion: HCG induces enormous changes in the gene expression of granulosa cells. Transcriptional profiling of granulosa cells is different in patients with GV oocytes compared with those without immature eggs. It seems that events that lead to hypoxia and vascularization of ovarian follicles could be implicated in obtaining immature eggs. These observations could help in understanding the mechanisms of HCG action and finding the pathways possibly involved in healthy follicular maturation.

Gene expression analysis on cumulus cells: a new tool for embryo quality assessment?

van Montfoort APA³, Geraedts JPM¹, Dumoulin JCM³, Stassen APM², Evers JLH³, Ayoubi TAY²

¹Department of Clinical Genetics, Academic Hospital Maastricht, The Netherlands; ²Department of Population Genetics, Genomics and Bioinformatics, University Maastricht, The Netherlands; ³Research Institute Growth and Development (GROW), Department of Obstetrics and Gynaecology, Academic Hospital Maastricht, The Netherlands

Objective: For optimal IVF results, especially after single embryo transfer (SET), the embryo with the highest implantation potential needs to be selected. Up till now, the established criteria based on embryo morphology and blastomere number have been used. Several new criteria are developed based on oocyte aspects. These, however, only represent morphology, while no information about the developmental capacity based on the folliculogenesis is given. Cumulus cells might be useful for developing additional criteria, as they surround the oocyte inside the follicle and therefore possibly reflect oocyte developmental potential. The aim of this study was to analyse the expression of genes in cumulus cells as an indicator of embryo viability and to define possible trivial processes in oocyte and embryonic development.

Materials/Methods: Gene expression in cumulus cells derived from 16 oocytes was analysed using 16 microarrays that each contained almost 55,000 oligonucleotide probe sets. Eight cumulus cell samples were derived from oocytes resulting in an early cleavage embryo (EC-CC) and another eight samples from oocytes resulting in a non-early cleavage embryo (NEC-CC). To exclude the effect of interindividual differences on gene expression, from four patients both an EC-CC and an NEC-CC sample were used and from two additional patients two EC-CC and two NEC-CC samples were used. The resulting embryos had all reached the 4-cell stage on day 2 with similar morphology grade. Twenty-five differentially expressed genes were selected to be verified by quantitative real-time PCR (qRT-PCR) on the original microarray samples as well as on 24 (12 EC-CC and 12 NEC-CC) extra independent samples.

Results: In cumulus cells from oocytes that result in early and late cleaving embryos, 611 genes were differentially expressed. These genes are mainly involved in cell cycle, angiogenesis, apoptosis, EGF, FGF and PDGF signalling, general vesicle transport and chemokine and cytokine signalling. With qRT-PCR, 18 (72%) of the 25 genes could be validated in the original samples used for the microarray analysis, and of these 12 (67%) could also be validated in independent samples. The most important validated genes (*CCND2*, *CXCR4*, *GPX3*, *CTNND1*, *DHCR7*, *DVL3*, *HSPB1*)

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and *TRIM28*) seem to point towards disturbed oocyte maturation and hypoxic intrafollicular conditions, which result in the delayed cleavage of the embryo.

Conclusion: The embryo development can be related to the expression of several genes in the cumulus cells. The differences in expression probably reflect a suboptimal intrafollicular environment, which influences the embryonic development. Although further research is needed, this opens up perspectives for a new embryo or oocyte selection parameter based on gene expression in cumulus cells.

Molecular mechanisms underlying ovarian follicle ageing: the potential role of advanced glycation end-products

Tatone C¹, Carbone MC¹, Marci R², Artini PG⁴, Secciani F⁵, Campanella MG⁵, Di Cola M¹, Amicarelli F³, Focarelli R⁵
¹Department of Biomedical Sciences and Technologies; ²Department of Experimental Medicine; ³Department of Basic and Applied Biology, University of L'Aquila, L'Aquila; ⁴Department of Reproductive Medicine and Child Development, Division of Obstetrics and Gynecology, University of Pisa, Pisa; ⁵Department of Evolutionary Biology, University of Siena, Siena, Italy

Objective: Ovarian functional decline with ageing has been so far deeply characterized in terms of accelerated depletion of the ovarian follicle pool and reduced ability to produce oocytes competent for fertilization and development (Tatone *et al.*, 2008). By contrast, potential causal factors of ovarian ageing remain to be established. In this respect, it is reasonable to take into account the main theories on ageing mechanisms according which damaged biomolecules, which can accumulate spontaneously and irreversibly as a side-effect of normal metabolism include the AGE (advanced glycation end-products), well-known markers of the ageing process (Yin and Chen, 2005). AGE form from the interaction of reactive carbonyl compounds such as reducing sugars, aldehydes and ketones with proteins, a reaction called 'glycation'. The complex interplay among AGE, oxidative stress and vascular damage make these compounds good candidates as factors involved in ovarian ageing. The present study has addressed the hypothesis of a possible involvement of AGE in ovarian ageing by using an animal model.

Materials/Methods: The study was carried out on ovaries of young (4–8 weeks old) and reproductively old (48–52 weeks old) female mice. Based on the use of a monoclonal antibody against AGEs formed by methylglyoxal (MG), a potent AGE precursor, we monitored the presence of these compounds in the ovaries by means of mono- and bidimensional electrophoresis, immunoblotting analysis and immunohistochemistry. Intraovarian MG concentrations were measured by HPLC. The activity of glyoxalase I, an enzyme with anti-AGE effect, was measured by using a specific biochemical assay.

Results: Our experiments revealed that the level of AGE was significantly higher in aged ovaries than in young ovaries, and that these compounds accumulated mainly in the ovarian stroma, vessels and follicular theca. A preliminary proteomic analysis of AGE products indicated that collagen might be one of the ovarian proteins mainly damaged by glycation during reproductive ageing. Moreover, we found that ageing ovaries, when compared with the ovaries of young mice, exhibited increased MG. These changes were associated with reduced activity of glyoxalase I.

Conclusion: The present results provide evidence that ovarian ageing is associated with the accumulation of AGE, suggesting a potential role for these molecules as molecular markers of this event. Based on their mechanism of action, it is reasonable to postulate that AGE could be responsible for direct and indirect alterations of the follicular microenvironment which can jeopardize the development of competent gametes in the aged ovary.

Genetic and epigenetic defects in germ cells

Epigenetics and chromosomal abnormalities in human oocytes

van den Berg I¹, Laven JSE², Galjaard R-J³, van Doorninck JH¹

¹Subdivision Reproductive Medicine, Department of Obstetrics and Gynaecology and Department of Clinical Genetics; ²Subdivision of Reproductive Medicine, Department of Obstetrics and Gynaecology; ³Department of Clinical Genetics, Erasmus Medical Centre, Rotterdam, The Netherlands

Objective: The generally low fertility in humans and increased subfertility with increasing age is mostly due to the number of chromosomal abnormalities in human oocytes. Identifying factors that cause aneuploidy in oocytes may offer possibilities to prevent these abnormalities *in vitro* or *in vivo*. A large amount of research has focused on chromosome aberrations in human oocytes, but little is known about the epigenetic factors that could cause these chromosomal aberrations. Epigenetic modifications such as histone methylation, phosphorylation and acetylation are well conserved through evolution, and play important roles in mitosis. Investigation of these modifications and their role in human meiosis could give more insight in meiotic chromosome segregation errors in human oocytes. Histone acetylation and subsequent deacetylation in oocytes is necessary for correct progress through meiosis. If disturbed, it may lead to aberrant segregation of chromosomes or chromatids due to decreased kinetochore function and imperfect spindle figures, resulting in aneuploidy. Mice oocyte data have shown a correlation of abnormal histone deacetylation and aneuploidy and a correlation between age, remaining histone acetylation and aneuploidy (Li *et al.*, 2006). The importance of histone modifications for aneuploidy formation in human oocytes is being investigated in the current study.

Materials/Methods: Surplus human oocytes were collected from the germinal vesicle (GV) up to the meiosis II (MII) stage (METC+, IC+). Oocytes were either used immediately or *in-vitro* matured until the MII stage. Cells were fixed with 4% paraformaldehyde, followed by an immunoassay with antibodies against acetylation of lysin 12 of histone 4 (H4K12ac) and alpha-tubulin for visualization of the meiotic spindle. Chromosome alignment was assessed by DAPI staining and spindle morphology was scored according to published criteria (Akiyama *et al.*, 2006).

Results: Mouse studies have shown that during maturation from GV to MII, chromatin in oocytes is actively deacetylated by histone deacetylases (HDAC). Blocking this process by an HDAC inhibitor is related to division errors such as chromosome non-disjunction which results in aneuploidy (Li

et al., 2006). In this study, we show that human oocytes have acetylated chromatin at the GV stage, and as in mice, this acetylation is removed during maturation. However, 55% of the human MII oocytes displayed remaining acetylation of H4K12. The effect of defective deacetylation was apparent on spindle morphology. While 80% of the oocytes with defective deacetylation had an abnormal formed spindle, correctly deacetylated oocytes showed a defective spindle in only 20% of cases. In addition, a maternal age effect on the deacetylation process was found during *in-vitro* maturation. Oocytes obtained from younger women showed less defective deacetylation than older women.

Conclusion: This study shows that remaining histone acetylation leads to incorrect formation of the meiotic spindle that will eventually result in aneuploidy. This indicates that chromosome abnormalities can be a consequence of defective deacetylation. Furthermore, the results demonstrate that advanced maternal age affects the deacetylation process during *in-vitro* maturation. Both findings are of great importance for IVM-IVF treatments, and more research on influences of epigenetic factors on oocyte and embryo quality is required.

Telomere elongation in spermatogenesis

Jorgensen P¹, Graakjaer J², Kolvraa S², Fedder J¹

¹Scientific Unit and Fertility Clinic, Horsens-Brødstrup Hospital; ²Clinical Genetics Departments, Vejle Hospital, Denmark

Objective: Telomere dynamics in spermatogenesis are important for reproduction, developmental biology and human ageing. Telomeres, which protect the DNA, shorten progressively with each somatic cellular division. When the telomeres reach a critical length, they will induce cellular senescence or apoptosis. The telomere length may consequently be an indicator of the replicative capacity of a cell. In germ cells, cancer cells and different stem cells, telomerase can elongate the telomere.

Materials/Methods: The complete telomere profile in different stages of the spermatogenesis has been studied in both mouse and rat, but never in humans until now. We have measured telomere length in spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids. This was done with a quantitative fluorescence telomere-staining procedure. Telomeres were stained using a telomere FISH kit with telomere-specific PNA-probes (DAKO, Denmark). The intensity of the fluorescent signal represents the length of the specific telomere and a specialized computer program (developed by DAKO, Denmark) was applied to convert the fluorescent signals into relative telomere lengths.

Results: The results showed that telomere dynamics resemble that seen in mouse and rat. Here, the telomere length shortens and a drop in telomerase activity from spermatogonia to round spermatids is seen. In elongated spermatids the telomerase activity is restored and the telomeres elongate, giving the spermatozoa long telomeres.

Conclusion: Telomere length in humans shortens from spermatogonia to round spermatids. It needs to be further examined what happens during the development from round spermatids to spermatozoa. Experiments are in progress to show what happens with the telomerase activity, apoptosis and the epigenetic structure of the telomeres during spermatogenesis. If telomeres are inherited from the parents, shorter telomeres transferred with ICSI will lead to shorter

telomere lengths in the offspring and thereby perhaps a shorter lifespan. Investigating this possibility will elucidate whether or not there is a potential risk in ICSI done with immature germ cells

Psychology

Psychological impact of infertility in healthy infertile (HI) males and their partners in comparison with spinal cord injured (SCI) males and their partners

Karimzadeh M¹, Salsabili N², Karimzadeh M¹

¹Azad University (Karaj), Human Science Faculty, Dept of Primary Education; ²Tehran University of Medical Sciences (Rehabilitation Faculty) Tehran, Iran

Objective: Parenthood is one of the major transitions in adult life for both men and women, and is associated with major emotional sequelae. Therefore, this study was designed to survey the psychological impact of infertility in healthy infertile male (HI male) and their partners in comparison with spinal cord injured male (SCI male) patients and their partners who presented to Mirzakouchack Khan Hospital, Shariati Hospital, and Koassar Infertility Centre in Tehran, Iran.

Materials/Methods: A descriptive analytical study was performed on 115 subjects (couples) who were referred to the IVF/ICSI programme at Mirzakouchack Khan Hospital, Shariati Hospital, and Koassar Infertility Centre in Tehran, Iran. Consent was obtained from 31 healthy infertile males with 20 of their partners and 34 SCI males with 30 of their partners, and they completed the symptoms checklist (SCL-90) and anxiety questionnaires for surveying psychological problems such as anxiety, depression, hypochondriasis, obsession, compulsion, aggression, paranoia and psychotism in relation to patients by cause of infertility, duration of infertility, age, education, and employment of the patients and their partners.

Results: The mean average age in the HI group was: males 33.37 and partners 28.3 years, and in the SCI group: males 31.68 and partners 28.4 years. This survey showed that the level of anxiety, depression, paranoia, compulsion, and psychotism is higher than normal average in all groups, which was not significant within their groups ($P > 0.05$). In SCI males, the level of depression, hypochondria, and compulsion increased significantly in relation to anxiety ($P < 0.05$), and employment directly decreased phobia among them ($P < 0.03$). In SCI partners, the duration of infertility and age significantly increased anxiety ($P < 0.019$) and hypochondriasis ($P < 0.033$); phobia and psychotism decreased by education ($P < 0.03$). In HI males, compulsion and psychotism were significantly related to a higher level of anxiety ($P < 0.001$), and employment was related to lower levels of aggression (0.001). With an increase in the duration of infertility in the HI partners, the level of anxiety, depression, hypochondriasis, and obsession became more prominent ($P < 0.05$) and the level of compulsion and aggression decreased significantly with education ($P < 0.05$).

Conclusion: Adequate attention to infertile patients and their partners' psychological aspects and their treatment with cognitive, behavioural therapies and social support before IVF/ICSI scheduling and treatment is of great importance, and will improve quality of life.

Sperm function

Membrane-based electrophoresis: an equally efficient method of sperm preparation for IVF and ICSI

Fleming S¹, Ilad R¹, Griffin A-M¹, Ong K¹, Wu Y¹, Smith H¹, Aitken J²

¹Westmead Fertility Centre, Department of Obstetrics and Gynaecology, Westmead Hospital, Sydney, NSW 2145; ²ARC Centre of Excellence in Biotechnology and Development, University of Newcastle, NSW 2308, Australia

Objective: A novel system of membrane-based electrophoretic filtration that isolates spermatozoa with significantly less DNA damage has recently been developed. The preliminary data suggest that it is a viable method of sperm preparation for IVF. However, it is not known whether electrophoretic preparation of spermatozoa is as effective a method of sperm preparation for good and poor semen samples, as utilized for IVF and ICSI respectively.

Materials/Methods: Treatment outcomes of 17 IVF patients and 10 ICSI patients were compared following sperm preparation by electrophoresis (Gradiflow CS-10; NuSep Ltd). Fertilization and cleavage rates, and embryo quality post-insemination (PI), were compared using two-sided ANOVA, a P -value < 0.05 being considered statistically significant.

Results: No significant difference between spermatozoa prepared for IVF or ICSI by electrophoresis was observed with respect to subsequent rates of fertilization (65 versus 63.5% 16 h PI), cleavage (94.5 versus 100% 40 PI), and top grade day 2 embryos (32.7 versus 22.5% 40 h PI) respectively. There are insufficient pregnancies to statistically compare the pregnancy (44.4 versus 25%) and implantation (36.4 versus 20%) rates for IVF and ICSI.

Conclusion: These results suggest that membrane-based electrophoresis is an equally reliable and efficient means of preparing spermatozoa for both IVF and ICSI.

Posters

Cryopreservation

Successful ongoing pregnancy after transfer of two vitrified-warmed blastocysts derived from ICSI with a vitrified-warmed oocyte and frozen-thawed spermatozoa

Kyono K, Nakajo Y, Nishinaka C, Hattori H, Takizawa T, Ota N, Saito Y, Kumagai Y
Kyono ART Clinic

Objective: Cryopreservation technique is very important for oocytes, spermatozoa and embryos in assisted reproduction. We report an ongoing pregnancy after transfer of two cryopreserved blastocysts transfer derived from cryopreserved oocyte and cryopreserved spermatozoon for the first time.

Materials/Methods: The patient was a 35-year-old woman. Her husband had been diagnosed with nonobstructive azoospermia. Testicular sperm extraction from the husband's bilateral testes was performed, but no spermatozoa were obtained. Nine mature oocytes were cryopreserved. After 5 months, the couple decided to pursue treatment using donor spermatozoa. Consequently, after transfer of a single fresh blastocyst from a vitrified-warmed mature oocyte and frozen-thawed donor spermatozoa, a healthy male baby was born on December 1, 2004. The couple visited our new clinic wishing for a second baby. The patient underwent ovarian stimulation using a gonadotrophin-releasing hormone (GnRH) agonist long protocol, and nine of 17 mature oocytes were retrieved on September 28, 2007. It was intended to use the same donor spermatozoa used in the previous pregnancy, since that was the couple's request. However, when we looked for the spermatozoa in the tank for ICSI, the sample could not be found, and it was discovered that the spermatozoa had already been discarded by mistake. The couple did not wish to use another sperm donor, and decided to have the oocytes vitrified. After 2 months, the couple decided to pursue treatment using donor spermatozoa. Nine vitrified oocytes were warmed and used for ICSI. A single blastocyst (Gardner's criteria, grade 2) was transferred, and four blastocysts were vitrified, but pregnancy was not achieved. On December 27, two vitrified and warmed blastocysts (4BB and 3BB) were transferred.

Results: A gestational sac with fetal heart movements was confirmed by ultrasonography, and the pregnancy is now in its 8th week.

Conclusion: To our knowledge, we are the first to report an ongoing pregnancy after transfer of two vitrified-warmed blastocysts derived from ICSI with vitrified-warmed oocytes and frozen-thawed spermatozoa.

In-vitro maturation of sheep cumulus-oocyte complexes follow vitrification by conventional and cryotop methods

Ebrahimi B¹, Valojerdi MR^{1,2}, Yazdi PE², Baharvand H³
¹Department of Anatomy, Faculty of Medical Sciences, Tarbiat Modares University; ²Department of Embryology, Royan Institute; ³Department of Stem Cells, Royan Institute, Tehran, Iran

Objective: This study was conducted to determine the optimal vitrification conditions for immature sheep oocytes using the conventional and cryotop methods.

Materials/Methods: Immature oocytes (cumulus-oocyte complex: COC) were harvested from slaughtered sheep ovaries by combination of aspiration and slicing methods.

Good quality isolated COC (fine granular cytoplasm and two or three compact layers of granulosa cells) were divided into three groups: control, conventional vitrification and cryotop vitrification. In the control group, COC were immediately transferred to in-vitro maturation (IVM) medium (TCM, 10% FBS, cysteamine, bFSL, bLH, kanamycin, Na pyruvate and Stradiol). In the vitrified groups, two-step vitrification was carried out as follow: equilibration by DMSO, EG, FBS and α -MEM and vitrification with DMSO, EG, sucrose, FBS and α -MEM. Vitrification was performed using conventional straws and cryotop. After a week vitrified COC were warmed in four steps: 1, 0.5, 0.25, 0 mol/l sucrose, FBS, α -MEM and 2 h after warming their viability was assessed morphologically with $\times 20$ inverted microscopes. Then COC were transferred to IVM medium and incubate for 20–24 h at 39°C and under 5% CO₂ tension. After maturation, COC of all groups were denuded and stained by Hoechst for nuclear stage determination.

Results: Data were confirmed by Kolmogorov-Smirnov normalization test, then numbers of healthy and degenerated COC were compared using the Tukey test. The mean percentage of healthy COC after vitrification and warming was highest in the cryotop group (81.32%) and also showed a significant difference between conventional and cryotop groups ($P < 0.05$). After maturation follow vitrification and warming, the percentage of healthy oocytes was higher in cryotop than conventional groups and there was a significant difference between conventional groups by cryotop and control groups ($P < 0.05$). Mature oocytes (MII) had the highest percentage in the cryotop group (50.02%) after control group (51.73%), and again the conventional group was significantly different from the control and cryotop groups.

Conclusion: According to the particular sensitivity of immature oocytes to freezing due to their unique biological characteristics, it seems that vitrification by cryotop can reduce cryoinjuries and increase the viability and post-thaw quality of sheep COC after vitrification by volume reduction and high speed freezing.

Evaluation of cell death in in-vitro matured follicles after vitrification of mouse ovary

Mazoochi T¹, Salehnia M², Valojerdi MR², Mowla SJ², Moghadam MF²

¹Department of Pathology, Kashan University of Medical Sciences, Kashan; ²Tarbiat Modares University, Tehran, Iran

Objective: This study was carried out to evaluate the effect of mouse ovarian tissue vitrification on follicular apoptosis. This experimental study was carried out on 12–14-day-old female mice (NMRI). Ovaries were vitrified with a solution containing ethylene glycol and apoptosis compared with non-vitrified ovaries in two steps.

Materials/Methods: In the first step, occurrence of apoptosis was evaluated using a combination of several techniques including morphological, ultrastructural, TUNEL and DNA laddering assay in vitrified-warmed and non-vitrified mouse ovaries. In the second step, expression of apoptotic genes (*Bax*, *Bcl-2*, *Fas*, *FasL*, *p53* and *Survivin*) was evaluated in isolated in-vitro matured follicles from vitrified-warmed and non-vitrified ovaries and compared with the same stage of follicles *in vivo*. Also, in order to determine the healthy and degenerated cells in antral follicles, flow cytometric analysis was performed in the three study groups.

Results: No sign of apoptosis was observed morphologically or by TUNEL technique and gel electrophoresis in either vitrified or non-vitrified ovaries. The survival and developmental rates of isolated preantral follicles from the vitrified ovaries was the same as non-vitrified groups. All evaluated genes were expressed in different sized in follicles in three groups of study, whereas Fas mRNA was not expressed in preantral follicles. The relative abundance of FasL and Bax mRNA to the β 2-m was similar in all groups, but Fas and p53 mRNA was strongly expressed in antral follicle vitrified groups compare with non-vitrified. The expression of Bcl-2 and Survivin 140 was lower in large preantral follicle in the vitrified groups compared with the non-vitrified samples. Flow cytometric analysis showed that the percentage of healthy cells in vitrified group was significantly lower than non-vitrified samples.

Conclusion: Vitrification of ovaries does not induce apoptosis just after warming. However, it has some minor effects on the apoptotic-related gene expression.

Comparison between cryodamage of human spermatozoa after freeze–thawing with a biological freezer or ultrarapid freezing (vitrification)

Hammadeh ME, Fischer-Hammadeh C, Rosenbaum P, Schmidt W

Department of Obstetrics and Gynaecology, University of Saarland, Homburg/Saar, Germany

Objective: The purpose of this study was to determine and compare the negative effects (cryodamage) on human spermatozoa (chromatin condensation, morphology, acrosome reaction, DNA strand breaks) after freeze–thawing with a programmed slow freezer or using vitrification technique (ultrarapid freezing).

Materials/Methods: Forty-one semen samples were obtained from men attending our andrology laboratory for semen analysis. After liquefaction, each semen sample was evaluated according to WHO guidelines, except for morphology, which was assessed according to strict criteria, and divided into two parts after the addition of the cryoprotectant. The first part was frozen using a programmed biological freezer (Planer) and the second part was frozen by means of vitrification. Smears were made before and after freeze–thawing to assess morphology, chromatin condensation (Chromomycin CMA3), acrosome integrity, which was assessed after staining with concanavalin-A-lectin, and DNA strand breaks (TUNEL test).

Results: The mean percentages of morphologically normal spermatozoa, chromatin condensed (CMA3), with intact acrosome and the mean percentages of DNA strand breaks in the native semen samples were 25.4 ± 18.9 ; 71.05 ± 8.71 ; 77.05 ± 7.34 and $84.33 \pm 7.71\%$ respectively. The mean percentages of these investigated parameters (exception morphology) decreased significantly after freeze–thawing using a programmed biological freezer to 26.95 ± 19.42 ; 44.11 ± 19.15 ; 52.48 ± 10.12 and $62.10 \pm 10.31\%$ and after freeze–thawing by means of vitrification technique to 26.62 ± 18.19 ; 43.62 ± 17.11 ; 52.57 ± 12.05 and 64.48 ± 10.49 respectively. No significant difference could be found between the mean percentage of cryoinjury after freeze–thawing by using a programmed biological freezer (Planer) or vitrification technique.

Conclusion: The mean percentage of condensed chromatin, acrosome integrity and DNA strand break decreased

significantly after freeze–thawing not only by means of vitrification, but also by using a programmed biological freezer. However, these decreases in the mean percentage of these parameters were similar using both techniques. Therefore, the vitrification technique could be recommended for freezing of good quality human spermatozoa.

Development of vitrified–warmed mouse embryos co-cultured with polarized or non-polarized uterine epithelial cells using sequential culture media

Azadbakht M¹, Valojerdi MR², Mowla SJ³

¹Department of Anatomy, Tarbiat Modarres University, Tehran and Department of Biology, Razi University, Kermanshah; ²Department of Anatomy, Tarbiat Modarres University, Tehran and Department of Embryology, Royan Institute, Tehran; ³Department of Genetics, Tarbiat Modarres University, Tehran, Iran

Objective: This study investigated the effects of the in-vitro co-culture of vitrified–warmed mouse embryos with non-polarized or polarized uterine epithelial cells using sequential culture media, on their development to blastocysts, blastocyst quality (blastocyst diameter and cell number), apoptosis and Bcl-2 or Bax gene expression.

Materials/Methods: There were three treatments, all of which used sequential culture media. The treatments were no co-culture (control), non-polarized or polarized epithelial cell monolayer co-culture in 24-well tissue culture plates. Mouse uterine epithelial cells were isolated enzymatically and were seeded either on the surface of the culture plate (non-polarized monolayer) or on a Millipore filter insert coated with extracellular matrix extract (polarized monolayer) that was then placed in the culture plate. Two-cell mouse embryos were vitrified by the closed pulled straw (CPS) method. Embryos were pretreated in 1.5 mol/l ethylene glycol, vitrified within in 5.5 mol/l ethylene glycol and 1 mol/l sucrose solution, and warmed in stepwise sucrose solution. The survived embryos were cultured in G-1 ver3 medium to the 8-cell stage, when they were randomly assigned to the treatments. The culture medium was G-2 ver3 during the treatment phase of the study. Significances of differences were evaluated by the one-way analysis of variance for continuous data.

Results: The epithelial cells cultured on Millipore filters became polarized and their morphology compared favourably with that of cells cultured on the surface of the culture plate. After 96 h on the treatments, the polarized monolayer had supported the development of significantly more hatched blastocysts (63.4%) than the non-polarized monolayer (42.7%) or the control (39.5%) culture treatments ($P < 0.05$). Co-culture resulted in the production of larger blastocysts and inner cell mass (non-polarized monolayer 3.4 ± 0.3 and 2.6 ± 0.3 , polarized monolayer 3.1 ± 0.2 and 2.8 ± 0.2) than the control culture (2.1 ± 0.3 and 0.5 ± 0.1), ($P < 0.05$). The polarized monolayer resulted in the production of blastocysts with significantly more cells (59.0 ± 2.8) than the non-polarized monolayer (40.8 ± 2.7) or the control (34.2 ± 2.7) culture treatments ($P < 0.05$). The proportion of blastocysts with apoptotic blastomere was higher for the control culture (100%) than for the non-polarized (76%) or polarized (75%) co-culture systems but the difference was not significant. Moreover, the apoptotic index was significantly higher in control blastocysts (9.2 ± 1.1) than in non-polarized (4.4 ± 0.8) or polarized (2.4 ± 0.4) co-culture ($P < 0.05$). The Bax mRNA expression was lower for the polarized co-culture

than the non-polarized co-culture and control treatments ($P < 0.05$); however, the relative abundance of Bcl-2 mRNA to the β -tubulin was similar for all treatments.

Conclusion: It is concluded that a co-culture system involving polarized uterine epithelial cells and sequential culture media is a promising method for the improvement of mouse embryo development *in vitro* after vitrification-warming, and may be able to rectify injuries resulting from vitrification-warming in embryos.

Embryo culture

Value of pronuclear morphology as a marker for development and competence of embryo in ICSI procedure

Elsraite O, Danfour M, Elmahaishi M

Faculty of Medicine-7th October University and Misurata Infertility Centre, Misurata, Libya

Objective: The relation between pronuclear zygote morphology and their fertilization potential and embryo developmental competence after intracytoplasmic sperm injection (ICSI) is an important issue, and has gained much attention recently due to its possible value in predicting implantation and pregnancy. This study was carried out to investigate a possible prognostic value of pronuclear morphology with four different pronuclear orientations on fertilization rate and embryo quality.

Materials/Methods: A total of 51 consecutive ICSI cycle (304 oocyte) were included in this study. All women were ≤ 40 years of age. All oocytes were culture individually in order to follow their developmental potential. Embryo quality was assessed using both pre-embryo pronuclear morphology (zygote scoring or Z-scoring) at the time of fertilization evaluation and standard day-3 embryo morphology (number of blastomeres and grading based on degree of fragmentation and blastomere size). The main outcome measures were early cleavage rate, quality of embryos.

Results: The Z-score distribution of 276 embryos was 34.3% Z1, 26.4% Z2, 23.5% Z3, and 15.8% Z4. Significantly higher proportion of zygotes with Z1 and Z2 types of pronuclear orientation underwent early cleavage and developed into grade I embryos compared with other types ($P < 0.001$).

Conclusion: Our results demonstrated that a combined evaluation of the Z-score and day-3 embryo morphology is highly predictive of embryo outcome after ICSI. The Z-score could be of great help in the selection of embryos for cultures extended to later stages or selection of embryo for freezing at 2PN stage, and for determination of the most suitable embryos and the number of embryos for transfer, thus achieving the optimal chance of conception while reducing the risk of high order multiple pregnancy.

Effect of LH-treated oviductal epithelial cell co-culture system on mouse pre-embryo development

Alipour H², Rastegarnia A², Yazdi PE¹, Nejad MRBE¹

¹Royan Institute, Tehran, Iran; ²Urmia Azad University, Urmia, Iran

Objective: As with co-cultures of embryos with oviductal epithelial cells, oviductal glycoprotein (OGP) is thought to promote early embryonic growth and development. Murine oviductal epithelium contains LH receptors, which function in the increase of synthesis of OGP. Therefore the LH treatment

of co-cultures should further increases embryo development and quality through OGP mediation. This study was designed to develop a new coculture system and assess the effect of LH using sequential media to promote development and increase the quality of 1-cell murine embryos through the 8–16-cell stage to morula and blastocyst stages.

Materials/Methods: Monolayers for co-culture were prepared from murine oviduct epithelial cells (MOEC) in DMEM/F12 medium and in-vivo fertilized 1- to 2-cell embryos were collected by flushing from superovulated NMRI mice. Co-culture media were treated with HCG as a surrogate for LH because of its stability and purity. Embryos were cultured on a MOEC monolayer in G1/G2 Ver3 drops as the control and drops containing LH as the experimental group. Development and quality rates were determined for all embryos daily and compared statistically. At the end of the cultivation period, differentially stained trophectoderm (TE) and inner cell mass (ICM) of expanded blastocysts from each group were examined microscopically.

Results: The embryos cultured on a MOEC monolayer treated with LH had a higher developmental rate than those of the control group. There was no statistically significant difference; still, the blastocysts from the LH-treated group, in comparison with the control group, had a significantly higher mean cell number.

Conclusion: These findings suggest that elevated peri-ovulatory LH concentrations may promote preimplantation embryo development. These results have important implications for assisted reproductive technologies in which cocultures are used to improve pregnancy rates. Murine oviduct epithelial cell co-culture system treated by LH could improve in-vitro preimplantation embryo development more in terms of quality (increasing blastocyst cellularity) than in terms of developmental rate.

Proteomic approach to different embryonic *Gallus gallus* developmental stages

Garcillan DA², Lopez C¹, Requena A¹, Bou C¹, Mínguez Y¹
¹IVI Madrid, Spain; ²Rey Juan Carlos University, Madrid, Spain

Objective: The chicken (*Gallus gallus*) embryo is one of the most widely used experimental model organisms and has been for many years the most advanced model organism suitable for experimental embryology. Moreover, the chicken represents the model system, which, permitting experimental intervention *in ovo*, most resembles other higher vertebrates. As such, it represents an important complement to mouse model systems (Gregory *et al.*, 1998). Measuring gene expression at the protein level is potentially more informative than mRNA analysis. In contrast with the genome, which is essentially static, a proteome is highly dynamic. Processes such as differentiation, cell activation, disease or invasive infections can all significantly change the relative protein repertory.

Materials/Methods: In order to understand the molecular mechanisms underlying the normal and abnormal development of the chicken, two-dimensional electrophoresis (2-DE) was used to construct a proteome reference map of different stages of development (Kawakami *et al.*, 2003). Proteins were separated by isoelectric focusing on immobilized pH gradient (IPG) strips, and by 11% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Protein identification was done by peptide mass fingerprinting with

matrix-assisted laser desorption/ionization–time of flight–mass spectrometry (MALDI–TOF–MS) (Agudo *et al.*, 2004).

Results: In all, four stages of embryonic development were compared (stages 13, 21, 29 and 40), and were detected about 212 spots that appears in different stages, or in different amounts in each studied stage. Some of these proteins are related with the embryo development, others with different diseases, and another group has never been described before in this animal model. These maps will be updated continuously and will serve as a reference database for investigators, studying changes at the protein level under different physiological conditions.

Conclusion: These results suggest that the proteomic approach is valuable for the study of the embryonic development.

Novel protein-free embryo culture medium supports sperm function, fertilization, embryo development, implantation and initiation of viable pregnancies

Ali J

King Fahad Medical City, Women's Specialized Hospital, CTHM Reprod Med Unit, Riyadh, Kingdom of Saudi Arabia

Objective: Present-day embryo culture medium (ECM) contains donor serum proteins that have the potential to transmit harmful pathogenic protein-bound agents to patients/babies/healthcare workers. The European Union urged cessation of use of non-uniform biological preparations (EU Tissue Directive No. 2004/23/EU) by April 2007. This investigation was to formulate an efficacious protein-free medium (PF medium) specific for human embryos.

Materials/Methods: Fifty experiments were performed using the mouse zygote assay to determine the individual tolerance levels/optimal concentrations of 50 chemical components that could serve as alternate energy substrates, inorganic salts, antioxidants, chelators, osmolytes, amino acids, vitamins, antioxidants and macromolecules. These findings were utilized to formulate different ECM. The best ECM were then evaluated and modified to PF medium during the course of another 23 experiments and tested for suitability and safety, specific for human embryos.

Results: In the mouse, 100% of 2- and 4-cell embryos developed in PF medium to blastocyst stage (95.5 and 100% respectively in control medium containing proteins; $P > 0.05$). In sibling human oocytes the fertilization rate in PF medium was similar to or better than in control commercially prepared embryo culture medium containing proteins (CECM) for both conventional IVF (85.3%, 116/136 versus 79.2%, 118/149 respectively; $P = 0.2352$) and ICSI (77.8%, 196/252 versus 69.4%, 175/252 respectively, $P = 0.0432$). Quality of day-2 embryos in PF medium was superior to CECM. The average blastomere number was significantly higher in embryos generated in PF medium than CECM (3.7 versus 3.4 respectively, $P = 0.0011$). Embryo grade was also significantly higher in embryos generated in PF medium compared with CECM (3.0 versus 2.8 respectively, $P = 0.0007$; embryo grading range: 4 = excellent; 1 = poor). Human day-2 embryos generated in PF medium resulted in viable pregnancies (48%, $n = 114$; all age groups; 55% $n = 95$ in women <39 years; as opposed to 33%, $n = 1515$ for all age groups combined for embryos generated in CECM). To date, more than 53 apparently normal children were born from embryos generated in PF medium. These results were comparable or better than those obtained with CECM.

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Conclusion: This long-term investigation resulted in the formulation of, for the first time, efficacious PF medium specific for human embryos. The PF medium eliminates the risk of disease transmission, is safe and chemically non-variable option in assisted reproduction. The formulation of the PF medium has created interest in other areas as well. The protein-free culture technology has ramifications in the meat and dairy industries, stem cell technology, cell and tissue transplantation technology, etc, which as in human assisted reproduction could benefit from the application of protein-free culture technology.

Morphological correlations between early cleaved embryos and day 2 embryos

Joanne C², Roux C², Blagosklonov O², Agnani G¹, Dellis X¹, Lagré P¹, Souquet C¹, Cadel A², Bailly A², Maillet R¹, Riethmuller D¹

¹CHU Besançon Gynécologie Obstétrique; ²CHU Besançon Biologie de la Reproduction

Objective: The aim of this study was to establish morphological correlations between early cleaved embryos and embryos on day 2 after follicular puncture.

Materials/Methods: A total of 508 early cleaved embryos were analysed 32 h and 34 h after follicular puncture. Morphological abnormalities corresponded to cytoplasmic fragmentation, asymmetry of the two blastomeres, multinucleation and presence of micronucleated blastomeres. Characteristics of a top quality embryo on day 2 were absence of multinucleated blastomeres, four or five blastomeres and $\leq 20\%$ anucleated fragments.

Results: A total of 285 early cleaved embryos were considered as normal on day 1, corresponding to 206 top quality embryos on day 2 (72%). Hyperfragmentation on day 2 was already observed on day 1 in 65% of the cases. However, 43% of the hyperfragmented embryos led to top quality embryos on day 2. Micronucleated blastomeres were observed in 64 cases. In these conditions, 37 top quality embryos and 22 multinucleated embryos were obtained on day 2. Only 15 multinucleated non-fragmented embryos came from normal early cleaved embryos ($n = 70$) and only one embryo with asymmetric blastomeres was observed on day 1.

Conclusion: Morphological analysis of early cleaved embryos provides useful information. However, a strict definition of cytoplasmic fragmentation should be adopted.

Impact of low oxygen tension in IVF/ICSI treatment on clinical pregnancy outcome. A prospective randomized study

Mette M, Novella-Maestre E, Bertram Lindenberg F, Lindenberg S

Nordica Fertility Clinic, Section for Reproductive Biology and ART

Objective: Oxidative stress has recently emerged as one of the most important factors that negatively affect the embryo viability in assisted reproductive techniques. Reactive oxygen species (ROS) can be produced intracellularly, originating from oocytes and embryos, or intracellularly from environmental factors. Many sources of ROS exist in the assisted reproduction laboratory, and all can increase ROS generation by the embryo. The most important external factor that may affect the gamete and embryo viability is pO_2 ; the in-vitro environment is relatively hyperoxic as compared with in-vivo conditions. The present study compares the culture of human ova using two culture systems with different oxygen

environment.

Materials/Methods: A total of 198 consecutive patients in a routine assisted reproduction unit were blindly allocated, the day before oocyte retrieval, to either culture in a closed culture incubator with humidified 5% O₂, 5% (5.2–5.5) CO₂ and 90% N₂ partial pressure or in the conventional culture incubator with humidified atmospheric air supplemented with 5% CO₂. The primary efficacy endpoint was clinical pregnancies in week 7 visualized by fetal heart movements by transvaginal ultrasound scanning. Secondary variable were biochemical pregnancies, number of good quality embryos, number of fertilized ova and number of ova retrieved.

Results: The age for the patients in the two study groups were the same (37.1 versus 36.8, SEM = 0.51 years), fertilization rate, cleavage rate and number of embryos rated as suitable for transfer were equal in the two culture settings; however, pregnancy rate (34 versus 21%) (positive β-HCG) and clinical pregnancy rate seen by ultrasound in week 7 (33 versus 19%) were found to be significantly higher in the closed culture incubator with low oxygen tension and stable temperature.

Conclusion: These findings have demonstrated that a stable low oxygen atmosphere including rigorous stabilization of the temperature and humidity in routine IVF laboratory work benefit the clinical outcome.

First-step supplementation of sequential embryo culture medium with synthetic organic buffers supports development of mouse embryos in an elevated CO₂ environment

Swain J, Pool T

Fertility Centre of San Antonio, Texas, USA

Objective: Manipulation of embryos during IVF imposes artificial stresses that may compromise development and quality. One such stress involves fluctuations in pH of culture media. Current culture media are pH buffered using bicarbonate, which gives the desired pH range when CO₂ levels are adjusted within the environment of the incubator. However, bicarbonate is a weak buffer and allows a dramatic rise in media pH when moved to the reduced CO₂ of room atmosphere for observation and handling. Therefore, improved buffering capacity of embryo culture media, more resistant to perturbations in pH, may improve embryo quality. Synthetic organic buffers offer a means by which to improve buffering capacity. Therefore, we examined effects of addition of two synthetic organic buffers, MOPS and HEPES, to the first step of a sequential embryo culture system on mouse embryo development and resulting blastocyst quality.

Materials/Methods: Utilizing P1 as a base/control medium, we supplemented with 25 mmol/l HEPES, 25 mmol/l MOPS, 50 mmol/l HEPES or 50 mmol/l MOPS. All media contained 25 mmol/l sodium bicarbonate and maintained a pH of 7.25–7.30 when cultured within the same incubator. Osmolarity of all media were adjusted to 280–290 through adjustment of NaCl levels. Frozen–thawed one-cell mouse embryos were cultured for 48 h in various P1 formulations and subsequently moved to Global medium for 48 h. Embryo development was assessed every 24 h and total blastocyst cell number determined.

Results: No differences were observed in embryo development between treatments within the first 72 h. Total blastocyst development was similar between all treatments (conP1: 88%, 25MOPS: 90%, 50MOPS: 84%, 25HEPES: 92%, 50HEPES: 92%). However, presence of 25 mmol/l

MOPS resulted in significantly higher rates of blastocyst hatching at 96 h compared with controls (54 versus 33%) $P < 0.05$, while other treatments, though higher, showed no significant differences (25HEPES: 50%, 50MOPS: 43%, 50 mmol/l HEPES 50%). Total blastocyst cell numbers were similar between all treatments (con: 52 ± 3.24 , 25MOPS: 53 ± 3.78 , 50MOPS: 55 ± 2.52 , 25HEPES 48 ± 2.91 50HEPES: 49 ± 1.91).

Conclusion: The presence of synthetic organic buffers to improve buffering capacity of traditional bicarbonate buffered media supports mouse embryo development when present during the critical first 48 h of culture and 25 mmol/l MOPS improves blastocyst hatching rates. Due to reduced capacity of oocytes to regulate intracellular pH, future studies will examine effects of enhanced extracellular buffering capacity on oocyte developmental competence.

Human embryo multinucleation on day 2 reduces markedly blastocyst development

Herrero L, de Souza AP, Pareja S, Losada C, Martinez M, Bou C, Minguéz Y
IVI Madrid, Spain

Objective: In the laboratory, embryo transfer and freezing are usually performed on day 3 of embryo development. Slow cleaved, multinucleated or fragmented embryos are cultured for a few days more, and even frozen if they reach blastocyst stage. The aim of this study was to assess, in a retrospective manner, the effect of the multinucleation on day 2 and the ability of these multinucleated embryos to reach blastocyst stage, as indicator of their implantation potential.

Materials/Methods: Between January and July 2007, 150 surplus embryos (study group) after transfer and freezing on day 3 were cultured until blastocyst stage. Surplus embryos were multinucleated and poor quality embryos were cultured 2–3 days more to try to freeze them for the patient if good quality blastocysts were obtained. Embryo quality was assessed in terms of number of blastomeres, percentage of fragmentation, symmetry between blastomeres and nucleation of the blastomeres. All the embryos were multinucleated on day 2. Embryo quality and blastocyst formation rate were compared with a control group composed by 225 good quality embryos, without multinucleation, from the sequential culture programme where embryos were cultured also until blastocyst stage to choose the best blastocyst for transfer. In all cases IVF/ICSI was carried out according to standard procedures. All embryos were cultured in IVF medium (VitroLife, Sweden) from day 0 to day 3, and CCM medium (VitroLife, Sweden) until embryo transfer or freezing on day 5/6. We also compared the blastocyst formation rate according to the number of multinucleated blastomeres on day 2 and the type of multinucleation (binucleation, trinucleation and micronucleation). Statistical analysis was performed using chi-squared and t-test.

Results: Development comparison between 150 multinucleated embryos on day 2 (study group) and 225 good quality embryos (control group) (see **Table 1** page S-16). Embryo quality and blastocyst formation rate were significantly lower in the study group compared with the control group. Blastocyst formation according with number of multinucleated blastomeres on day 2 was also analysed. One binucleated blastomere embryos achieved 20% of blastocyst development rate, significantly lower than no multinucleated embryos (control group: 46.2% blastocyst,

$P < 0.01$). When one trinucleated blastomere was observed, the percentage of embryos reaching blastocyst stage was only 14.28 ($P < 0.01$ comparing with control group) and if there were micronucleation (one cell with ≥ 4 nuclei) or more than one cell multinucleated no blastocyst formation was achieved.

Conclusion: The presence of multinucleated blastomeres on day 2 embryos is clearly correlated with a decreased blastocyst development rate. The higher number of nuclei per blastomere, the lower blastocyst development rate.

Table 1.

	Study group	Control group	P-value
<i>n</i>	150	225	
No. blastomeres D2 (mean \pm SD)	3.36 \pm 1.35	3.76 \pm 1.01	0.001
% fragmentation D2 (mean \pm SD)	9.37 \pm 6.77	7.64 \pm 6.71	0.01
Symmetry on D2 (mean \pm SD)	1.79 \pm 0.63	1.62 \pm 0.52	0.004
No. blastomeres D3 (mean \pm SD)	6.35 \pm 1.8	7.07 \pm 6.76	0.0005
% fragmentation D3 (mean \pm SD)	10.9 \pm 6.7	8.15 \pm	0.0005
Symmetry on D3 (mean \pm SD)	2 \pm 0.55	1.79 \pm 0.52	0.001
Morulas on day 4 (%)	18	36.8	<0.0001
Arrested embryos on day 4 (%)	26	17.77	NS
Blastocyst development (%)	17.6	46.2	<0.0001

Slow human embryos on day 2: its ability to reach blastocyst stage

de Souza AO, Herrero L, Losada C, Pareja S, Alonso M, Bou C, Minguez Y
IVI Madrid, Spain

Objective: In the laboratory, embryo transfer and freezing are usually performed on day 3 of embryo development. Slow cleaved, multinucleated or fragmented embryos are cultured few days more, and even frozen if they reach blastocyst stage. The aim of this study was to evaluate the development of slow embryos on day 2 (2–3 cells), and the ability to reach blastocyst stage as indicator of implantation potential.

Materials/Methods: Between January and July 2007, we studied 152 slow embryos on day 2 (study group). They were cultured until day 5/6 and those that achieved a proper blastocyst morphology (visible inner cell mass and even trophoctoderm) were frozen. Embryo quality was assessed in terms of number of blastomeres, percentage of fragmentation, symmetry between blastomeres and nucleation of the blastomeres. Embryo development was compared with 225 good quality embryos on day 2 (control group), resulting from sequential culture IVF cycles (embryo transfer on day 5/6) performed in the same period of time. All embryos were cultured in IVF medium (VitroLife, Sweden) from day 0 to day 3, and on day 3 they were transferred to CCM medium (VitroLife, Sweden) until embryo transfer or freezing. Blastocyst formation rate was also compared according

S-16

to the cleavage rate between day 2 and day 3. Statistical analysis was performed using chi-squared and t-test.

Results: Table 2 below shows statistically significant differences in all studied parameters. We also compared embryo development (number of frozen blastocysts) depending on the number of cells on day 2 and day 3. No blastocysts resulting from embryos cleaved from 2 to 4 cells on day 2 and 3 respectively were frozen. However, 28.5% of blastocysts, resulting from embryos cleaved from 2 cells on day 2 to 7 to 9 cells on day 3, were cryopreserved in day 5/6 (comparable data with control group). Embryos cleaved from 3 cells on day 2 to ≥ 5 cells on day 3 did not show a statistically significant difference with control group in blastocyst development rate.

Conclusion: The data suggest that only 14% of slow embryos reach blastocyst stage. Embryos with 2 and 3 cells on day 2 and a good cleavage to day 3 show a blastocyst formation rate similar to control group.

Table 2.

	Study group	Control group	P-value
No. of embryos	152	225	—
Number of blastomeres on day 2 (mean \pm SD)	2.34 \pm 0.47	3.76 \pm 1.01	<0.0001
% of fragmentation on day 2 (mean \pm SD)	11.4 \pm 10.83	7.64 \pm 6.71	<0.0001
Symmetry of blastomeres on day 2 (mean \pm SD)	1.7 \pm 0.62	1.62 \pm 0.52	<0.0001
Number of blastomeres on day 3 (mean \pm SD)	5.36 \pm 1.12	7.07 \pm 6.76	<0.0001
% of fragmentation on day 3 (mean \pm SD)	12.7 \pm 9.3	8.15 \pm	<0.0001
Symmetry of blastomeres on day 3 (mean \pm SD)	1.97 \pm 0.49	1.79 \pm 0.52	<0.0001
Morula on day 4 (%)	7.03	3.76 \pm 1.01	<0.0001
Arrested embryos on day 4 (%)	24.21	17.77	<0.0001
Blastocysts (%)	14.06	46.2	<0.0001

Human embryos with visible uninucleated blastomeres are not associated with chromosomally normal embryos

Mifsud A, Herrero L, Muñoz J, Minguez Y, Agudo D, Requena A
IVI-Madrid, Spain

Objective: This study assessed whether the uninucleated cell status on day 2 of embryo development is correlated to a genetically normal embryo.

Materials/Methods: We have analysed a total of 89 embryos from 21 patients attending our PGD programme. The mean age of the patients was of 40 \pm 2.7 years, and advanced maternal age was the main indication for PGD (85.71%). The embryo biopsy was performed by laser in all cases (OCTAX) and the analysed chromosomes were 13, 15, 16, 18, 21, 22, X and Y chromosomes. The nucleated status was assessed on day 2 (48 h post-insemination) of embryo development using a high-power inverted microscope. The embryos were classified as uninucleated, multinucleated, and invisible nucleus. Statistical analysis was carried out by chi-squared test.

Results: A total of 68 embryos (76.4%) had chromosomal aberrations versus 19 embryos (21.35%) that were normal. Two of the embryos were undiagnosed. The embryos had an

average of 4.01 ± 0.6 and 7.7 ± 1.01 blastomeres on day 2 and day 3 respectively. In all, 72.06% of abnormal embryos had uninucleated blastomeres. A similar percentage (73.68%) was found among the genetically normal embryos. The comparison between these two percentages is not statistically significant ($P = 0.972$)

Conclusion: The uninucleated status of the embryos evaluated is not related to chromosomally normal embryos. We could conclude, at least in this group of patients, that the morphological status is not a predictive factor of the genetics of the embryo, and therefore the PGD is strongly recommended. Although the study has been performed in patients with advance maternal age it will be interesting to analyse if a similar scenario occurs in younger patients.

Is early cleavage status an additional criterion in the selection of day 2 embryos?

Joanne C², Roux C², Dellis X¹, Agnani G¹, Lagré P¹, Blagosklonov O², Colin A¹, Bailly A², Mauny F³, Riethmuller D¹, Maillet R¹

¹CHU Besançon Gynécologie Obstétrique; ²CHU Besançon Biologie de la Reproduction; ³CHU Besançon Département d'Information Médicale;

Objective: To demonstrate that early cleavage status is a valuable addition to existing embryo selection parameters, the study focused on the concept of top quality embryo.

Materials/Methods: A total of 277 consecutive transfers of two embryos were analysed. The routine practice during the studied period involved the transfer of two embryos. Embryo selection was based on day 2 morphological criteria. The characteristics of a top quality embryo were absence of multinucleated blastomeres, four or five blastomeres and $\leq 20\%$ of anucleated fragments. Three subgroups were identified according to the number of transferred top quality embryos. Non-early cleaved transferred embryos from a cohort with early cleaved embryos were distinguished from early cleaved transferred embryos and from non-early cleaved embryos from a cohort with non-early cleaved embryos. Clinical pregnancy rate (CPR) and twin rate were assessed.

Results: The best results were observed when two early cleaved top quality embryos were transferred ($n = 107$), 72 clinical pregnancies (67%) were observed in these conditions. The fact that only one top quality embryo was transferred did not modify the results. A cohort effect was identified when early cleavage was considered. Poorer results were observed when no early cleaved embryo could be obtained (CPR 33 versus 63%, $P < 0.005$). Early cleavage estimation was particularly interesting when no top quality embryo could be obtained. Twin rate was not modified either by the number of top quality transferred embryos or by the presence of early cleaved embryos. The higher twin rate (42%) was observed when no early cleaved embryo was obtained. The proportion of early cleaved embryos modified the twin rate.

Conclusion: Early cleavage is an additional prognostic factor. However, parameters other than the status of the transferred embryos should be taken into account in order to improve transfer strategy.

Chromosomal status of uni-pronucleated zygotes

Ramírez JM¹, Bronet F¹, Grassa LH¹, de Souza AP¹, Martínez IC¹, Barragan MA¹, García-Velasco JA^{1,2}

¹IVI-Madrid, C/Santiago de Compostela 88-bajo, 28035-Madrid; ²Rey Juan Carlos University, Spain

Objective: It is widely described that fertilization was determined by observation, at 16–19 h post-insemination/injection, of two polar bodies (PB) and two pronuclei (PN). However, since 2–3% of zygotes only appear 1PN, it could be because the oocyte has been parthenogenetically activated (without spermatozoa participation) or due to the asynchrony in appearance/disappearance of PN. The aim of this study was to assess the chromosomal status of 1PN zygotes that achieved top embryo quality on day 3, in terms of ASEBIR criteria (Association for the Study of Reproduction Biology Spain). This results were compared with a control group consisted of sex linked disease patients.

Materials/Methods: A total of 182 uni-pronucleated zygotes were cultured until day +3, 151 from ICSI and 31 from conventional IVF. We performed a monitoring of embryo quality according to ASEBIR criteria. Before culture in HTF media, we obtained 46 TQE: 36 from ICSI and 10 from IVF. The zona pellucida were removed by using Tyrode's acid and then we left the embryos 5 min in Ca¹⁺ and Mg²⁺ free media to separate the cells. At once, the nuclei of each cell were fixed with carnoid (methanol: acetic acid (3:1)) in a slice. Later, FISH analysis of 13, 15, 18, X and Y chromosomes was performed. Statistical analysis was performed by *t*-test and Fisher's exact test.

Results: The results are shown in **Table 1** below.

Conclusion: We observed differences, but not statistically different, among IVF and ICSI groups, either in percentage of TQE in particular, or percentage of total and normal diploid embryos. Comparing normal diploid embryos versus control group we get significant differences versus ICSI group ($*P < 0.0001$), but not versus IVF group. Therefore, we deduced that single pronucleated zygotes coming from IVF cycles had similar proportion of chromosomally normal embryos than control group, while those that coming from ICSI cycles had much less proportion. According to the results, using TQE coming from zygotes uni-pronucleated in IVF cycles for freezing or even transferring (if it was necessary) could increase the outcome of the cycles.

Table 1.

	IVF	ICSI	Control group
Total zygotes 2 PB–1PN	31	151	–
Age (mean)	33.9	31.8	–
TQE (%)	10 (32.2)	36 (23.8)	108
Analysed embryos	9	33	108
Analysed cells/embryo (mean)	3.9	4.5	1.6
Total diploids (%)	7 (77.8)	10 (30.3)	103 (95.4)
Normal diploids (%)	6 (66.7)	8 (24.2)*	79 (73.2)*
Haploids (%)	1 (11.1)	19 (57.6)	1 (0.93)
Mixoploids (%)	1 (11.1)	4 (12.1)	0 (0)
Total mosaics (%)	1 (11.1)	6 (18.2)	3 (2.7)
Embryos with Y chromosome (%)	5 (55.5)	5 (15.2)	49 (45)

Implantation also occurs in multinucleated embryos

Gadea B, Escribá MJ, Grau N, Galán A, Gámiz P, De Los Santos MJ

Institut Universitari-Instituto Valenciano de Infertilidad, Valencia, Spain

Objective: Multinucleation is a relative common event in human embryos and it is generally associated with poor embryo development and IVF outcome. However, it is known that multinucleated embryos also implant and give healthy births. The aim of this study was to determine whether we can identify morphological parameters in multinucleated embryos able to predict both implantation and ongoing pregnancies.

Materials/Methods: Retrospective analysis of 65 transfers with multinucleated embryos of IVF/ICSI cycles. Embryo transfers were divided into two groups: group A (n = 34) had 100% implantation rate and group B (n = 31) had 0% of implantation rate. The mean (\pm SD) age of patients included in this study was 29.7 ± 4.4 years. Embryos were scored by the following morphological parameters: number of blastomeres, fragmentation, type of fragmentation, symmetry and percentage of multinucleation on day 2 and day 3 of development. For statistical analyses, the chi-squared, Student's t-test and ANOVA were used. P-values < 0.05 were considered significant.

Results: No differences were found in terms of mean number of blastomeres, percentage of fragmentation, type of fragmentation, symmetry and percentage of multinucleation between embryos from both groups A and B on day 2 (3.47 versus 3.52, $P = 0.5$; 7.41 versus 11.61, $P = 0.3$; 52.3 versus 47.7%, $P = 0.2$; 52.3 versus 47.7%, $P = 0.5$; 35.88 versus 40.59, $P = 0.1$ respectively). Regarding day 3 embryos, neither mean number of blastomeres, symmetry, nor percentage of multinucleation showed statistical differences (7.38 versus 6.42, $P = 0.1$; 52.3 versus 47.7%, $P = 0.3$; 36.6 versus 34.4%, $P = 0.6$ respectively). However, percentage of fragmentation was statistically higher in multinucleated embryos that did not implant compared with embryos that did implant (16.0 versus 7.12; $P = 0.04$). In addition, the worse type of fragmentation was more frequent on embryos that did not implant (61.3 versus 38.7%; $P = 0.026$). Furthermore, in the group of multinucleated embryos that implanted we could not identify any predictive parameters for ongoing pregnancies.

Conclusion: Our observations support the idea that multinucleated embryos are able to successfully implant. In addition, the percentage and type of fragmentation were the best predictive morphological parameters that embryologist can use as a tool to select the multinucleated embryo with higher chance of implantation as occurs with non-multinucleated embryos.

Efficiency of non-selected pronuclear stage embryo transfer

Ciriminna R, Barbaro R, Agrifoglio V, Napoli P, Palermo R
Associazione Medici e Biologi per la Riproduzione Assistita, Palermo, Italy

Objective: From March 2004, Italian law on Assisted Reproduction Techniques imposed major limitations that restrict the selection of embryos for transfer after assisted reproduction. No more than three embryos may be created and transferred, all of the fertilized oocytes or embryos must be transferred in a single procedure, and any kind of embryo selection or cryopreservation is forbidden. Zygote transfer has previously been reported to succeed in creating viable

pregnancies, and therefore, in view of the legal restrictions, we evaluated the efficiency of transferring non-selected zygotes as compared with the transfer of non-selected cleavage stage embryos.

Materials/Methods: This study retrospectively analysed data from 286 patients who initiated a total of 347 cycles of ICSI in a 43-month period, from March 2004. Cycles were divided in two groups: group 1 (G1): 127 cycles in which zygote transfers were planned 18–22 h after ICSI and group 2 (G2): 220 cycles in which cleavage stage embryo transfer was planned 44–48 h after ICSI. For the analysis of the results, each group was divided into two subgroups according to female age: G1a: (84 cycles) age ≥ 38 years and zygote transfer; G1b: (43 cycles) age <38 years and zygote transfer; G2a: (109 cycles) age ≥ 38 years and embryo transfer; G2b: (111 cycles) age <38 years and embryo transfer. Chi-squared test was used for comparisons between the groups.

Results: The overall pregnancy rate (PR) (27.0 versus 35.4%) and implantation rate (IR) (14.0 versus 17.4%) between G1 and G2 was not statistically different. Similarly, comparing G1a versus G2a (PR: 15.5 versus 20.2% and IR: 7.0 versus 8.0%) as well as G1b versus G2b (PR: 48.8 versus 50.4% and IR: 21.8 versus 25.5%) no statistically significant differences in pregnancy rate and implantation rate were found.

Conclusion: The results suggest that the efficiency of zygote intrauterine transfer on day 1 in terms of implantation and pregnancy rates does not differ from results after transfer of cleavage-stage embryos on day 2. Therefore, within restrictions that prevent any potential selection of embryos for transfer, embryo transfer at the earliest stage may represent a valid and effective time-reducing strategy for an assisted reproduction facility. However, a randomized prospective trial is needed to confirm our observations.

Clinical application of Spanish embryo classification

Prados F, Pérez-Bermejo G, Pinto S, Collado O, Sánchez-Rivera M, Bruna I
Hospital de Madrid-Montepríncipe, Madrid, Spain

Objective: The Spanish Association of Embryologists (ASEBIR) has recently proposed the use of a consensus new embryo classification. This classification has been based on contrasted publications concerning the relationship between embryo morphology and implantation potential. The implementation of this system will allow more accurate definition of embryos to other colleagues. Embryos were evaluated and allocated into four categories: grade A, B, C and D in decreasing morphological quality. This work describes the use of this new classification.

Materials/Methods: The features selected to organize the embryos into these categories will be described. This presentation includes the 4286 embryos produced in the 810 homologous oocyte cycles performed in our clinic from January 2005 until October 2007 to patients younger than 40. The morphological evaluation, transfer and freezing were performed either on day 2 or day 3.

Results: The implantation rates of each embryo category in homogeneous transfers were: 53.9% (138/256) for grade A; 46.0% (57/124) for B; 26.9% (54/201) for C and 14.0% (13/93) for D ($P < 0.05$). In all 20, 6% of the 3940 embryos evaluated on day 2 were grade A; 18.1% grade B; 26.4% grade C and 34.9% grade D. Among the 1489 embryos that were evaluated on day 2 and again on day 3, 12.8% were classified as grade A; 15.2% as grade B; 27.8% as grade C and 44.2%

as grade D. The survival rate after thawing (before November 17, 2007) of grade A frozen embryos was 82.8% (120/145); 82.5% (132/160) in grade B; 78.4% (149/190) in grade C and 76.5% (13/17) in grade D embryos. The percentage of thawed embryos which implanted was 42.3% (22/52) in the case of grade A; 28.1% (16/57) grade B; 13.1%, (14/107) grade C and 0% (0/7) grade D ($P < 0, 05$)

Conclusion: The four-category system proposed by ASEBIR for the morphological classification of embryos correlates accurately with their probability of implantation. The most numerous group of embryos were grade D, followed by C; grade B was the smallest group of embryos evaluated on day 2, while grade A was the smallest when the evaluation was on day 2 and again on day 3. Grade D embryos have a low but not negligible likelihood of implantation in fresh transfers. The creation of a 'grade E' category could be helpful to describe non-viable embryos. The frozen embryos of grades A and B showed significantly better implantation rate per thawed embryo than grade C embryos. To date we have not observed the implantation of any frozen grade D embryo. The high implantation potential of grade A embryos in patients younger than 40 (53.9%) together with the probability of survival and implantation of grade A and B frozen embryos can be useful to perform elective SET without compromising the cumulative pregnancy rates.

Gametes and stem cells

Co-culture of Sertoli cells and human spermatogonia stem cells for developing an in-vitro culture system capable of supporting human spermatogonia stem cell colonization

Mmirzapour T^{1,2}, Movahedin M², Ibrahim TA¹, Khansarinejad B³, Kkoruji M², Nowroozi MR⁴, Radkhah K⁴, Rafeian S⁴

¹University Putra Malaysia-Institute of Bioscience; ²Department of Anatomical Sciences, School of Medical Sciences, Tarbiat Modares University; ³Department of Virology, School of Medical Sciences, Tarbiat Modares University, Tehran; ⁴Research Centre in Fertility and Infertility Hospital of Imam Khomeini, Iran

Objective: Up to 20% of couples worldwide are infertile. Male factor infertility is responsible for reduced pregnancy rates in 40–60% of infertile couples. There is increasing evidence that the transition from undifferentiated to differentiating spermatogonia is a point of control during spermatogenesis (Roosij, 1998). Widely different conditions can lead to blockade of this differentiation step and finally lead to maturation arrest (Meachem, 2001). Isolation, proliferation and in-vitro colonization of spermatogonia stem cells will allow powerful new approaches in biological basis for male reproduction and for treating selected causes of male infertility.

Materials/Methods: Biopsy of testis was obtained from patients with arrest in one of process of spermatogenesis (maturation arrest). Samples were minced into small pieces and were suspended in DMEM, which contained collagenase/dispase, trypsin, hyaluronidase and DNase, with shaking at 32°C. After three washes in DMEM and removal of most of the interstitial cells, a second digestion step was performed in DMEM by adding fresh enzyme. A mixed population of the cells obtained by enzymatic digestion were placed on

lectin-coated dishes. The spermatogonia that remained in suspension were collected and kept at 32°C in the presence of FBS. After 2 days, Sertoli cells formed a confluent layer, then spermatogonial cells were co-cultured on top of them. Assay of the spermatogonial cell-derived colonies was commenced after 7 days of co-culture and carried out every 3 days after the appearance of colonies during culture by an inverted microscope. The numbers of Sertoli and spermatogonial cells were determined with a haemocytometer. Cell viability was evaluated by means of the dye exclusion test.

Results and Conclusion: The cell population obtained from the seminiferous tubules of human testes contained mostly two different cell types with different sizes and morphology. The first type was 7.5–8.2 µm in diameter and had an irregular outline with a granular appearance. Larger than the first one, the second type had a diameter of 14–16 µm, a spherical outline and two or three eccentrically placed nucleoli. The first type proliferated and created a monolayer of cells, whereas the other type created a colony after proliferation. Sertoli cells generate and maintain the cytoarchitecture of the germinal epithelium, produce nutrients that provide energy substrates to the germ cells and, in the primate, represent the only cellular component of the blood–testis barrier (Dym, 1977). In conclusion, we sought to demonstrate that Sertoli cells could influence human spermatogonial proliferation *in vitro*.

Assessment of morphological and functional changes in the mouse testis and epididymal spermatozoa following unilateral and bilateral cryptorchidism; a mouse model

Absalan F, Movahedin M, Mowla SJ
Tarbiat Modares University, Tehran, Iran

Objective: This study was designed to evaluate the long-term effect of experimental bilateral and unilateral cryptorchidism on sperm parameters and structure of mouse testis.

Materials/Methods: To induce bilateral and unilateral cryptorchidism model, immature mice (age under 2 months) were anaesthetized and a small incision was made in the abdominal skin and peritoneum, then the fat pad at the upper end of testis was sutured to the peritoneum. Testes were removed after 2, 4, 6 and 8 weeks after surgery, weighed and processed for light microscopy study. Weight of testis, spermatogenic cell numbers, tubular ectasia (rate of tubular lumen comparing to the germ cell layer) as well as epididymal sperm parameters were measured.

Results: Spermatogenesis was arrested and the testicular weight and seminiferous tubular diameters were significantly reduced in the bilateral undescended testis compared with unilateral undescended testis and the control mice. However, complete depletion of seminiferous tubules and absence or sloughing of germ cells were not found in all the animals. Spermatocytes and spermatids were the main type of germ cells undergoing apoptosis in all groups.

Conclusion: In general, high temperature caused a decrease in all analysed parameters except spermatogonial cell number, probably due to the apoptosis and these changes significantly increase in bilateral groups compared with unilateral groups. It is suggested that although apoptotic cell death induced by bilateral cryptorchidism might be affected by changes in systemic factor, apoptosis increase in male germ cells after unilateral cryptorchidism regulated by local testicular factors. This model is also suitable for enrichment of spermatogonial stem cells.

BMP4 supports the proliferation and viability of mouse embryonic stem cell in a dose-dependent manner

Makoolati Z¹, Movahedin M¹, Forouzandeh-Moghadam M²

¹Department of Anatomy, Medical Sciences Faculty;

²Department of Biotechnology, Medical Sciences Faculty, Tarbiat Modares University, Tehran, Iran

Objective: Bone morphogenetic proteins (BMP) are signalling molecules from the transforming growth factor β (TGF β) superfamily that signal through heteromeric complexes composed of type I and II serine–threonine kinase receptors. Binding of BMPs to their receptors induces phosphorylation of the BMP-specific Smads (Smad 1, 5, 8). Phosphorylated Smads1/5/8 then associate with Smad4, translocate into the nucleus, and activate the transcription of BMP target genes. With regard to the importance of BMPs roles in the formation, development and function of various vital systems during fetal life, the aim of this study was to evaluate the effect of different doses of BMP4 on the viability and proliferation of CCE mouse embryonic stem (ES) cells.

Materials/Methods: ES cells were trypsinized and cell suspension was prepared. The cells were counted and cultured in 96-well microplate. Each well of this plate contained 3×10^4 cells in 20% FCS in DMEM media. The cells incubated for 1 day, washed with PBS and cultured in DMEM containing different doses of BMP4 (1, 5, 25, 50 and 100 ng/ml) as experimental groups. Control group was cultured in BMP4-free medium. ES cells incubated at 37°C overnight, washed with PBS, trypsinized and cell suspension was prepared separately from each well. In order to investigate the viability and proliferation rates of ES cells, staining with trypan blue and counting were done. The mean number of whole cells and living cells were considered as proliferation and survival rates respectively. Data analysis was done using the ANOVA test.

Results: No significant differences were found between the mean number of whole cells in different doses ($P = 0.18$), but the mean percentage of living cells showed that BMP4 in 5 and 100 ng/ml concentrations had the best and the worst effects on the viability of ES cells respectively (65.56 versus 27.24%).

Conclusion: Evaluation of proliferation and viability rates using cell count and data analysis showed that addition of 5 ng/ml BMP4 increased the proliferation and viability rates of CCE ES cells, whereas high doses decreased these criteria. This suggests that different doses of BMP4 signalling may have different effects on ES cell behaviour. In other words, the proliferation and viability rates were associated with addition of BMP4 in the culture in a dose-dependent manner. BMP4 protein may phosphorylate Smad proteins in ES cells and consequently ES cells acquiring Smad (1, 5, 8) activation above a certain threshold, and so this concentration induced the expression of a particular set of genes involved in proliferation and viability. Thus, it is likely that different set of genes may be induced at different concentration of BMP4. As 5 ng/ml BMP4 had the best effects on the proliferation and viability, it is proposed as the best dose among different doses that studied in this research.

Implantation

Comparison of alkaline phosphatase activity of endometrial epithelium in hyperstimulated mice after administration of progesterone and oestrogen plus progesterone

Dezfolian AR¹, Niknafs B², Afshar F²

¹3-Department of Histology, Medical Faculty, Ahvaz University (Medical Sciences), Ahvaz; ²Department of Anatomy, Medical Faculty, Tabriz University (Medical Sciences), Tabriz, Iran

Objective: The implantation window was characterized by high alkaline phosphatase (ALP) activity in uterine epithelium and stroma. The enzyme activity was dependent on uterine cycles and endometrial phase. However, ALP activity arising from replacement hormones, e.g. LH, in hyperstimulated mice is unknown. In this study, ALP activity was studied for five groups following application of different luteal support hormones

Materials/Methods: Female mice were superovulated by PMSG and HCG and then were mated with vasectomized mice; the mice were divided into two groups; experimental and control group. There were three experimental groups; hyperstimulated mice were given four consecutive daily injection of progesterone, (P group), oestrogen (E group), oestrogen + progesterone (E + P group), and vehicle as a hyperstimulated control group. Natural control group mice were mated with vasectomized mice without any hyperstimulation. The uteri of all groups were collected after 4 days of hormone injection at window implantation phase. The uteri were prepared for alkaline phosphatase (ALP) staining.

Results: The results showed ALP activity observed in all groups. ALP activity of P + E group similar to E group, hyperstimulated group showed low staining compared with E + P, E and control group. The control group expressed much more ALP activity in relation to P and hyperstimulated groups.

Conclusion: In conclusion, hyperstimulation caused altered ALP activity. ALP activity showed significant differences between P and control groups. Although hyperstimulation decreased ALP activity in all groups compared with control group. P + E and E groups showed increased enzyme activity. This study suggested P + E injection at luteal phase in hyperstimulated cases instead of P injection.

Morphological changes of endometrial epithelium in hyperstimulated mice after application of progesterone and oestrogen plus progesterone

Niknafs B¹, Afshar F¹, Dezfolian AR²

¹Department of Anatomy, Medical Faculty, Tabriz University (Medical Sciences), Tabriz; ²Department of Histology, Medical Faculty, Ahvaz University (Medical Sciences), Ahvaz, Iran

Objective: The implantation window was characterized by changes in uterine epithelium and stroma. Morphological alterations of endometrial epithelium, as a contact site of embryo at implantation phase, by replacement hormones at luteal support phase in hyperstimulated mice are in debate. In this study, the morphological alterations were studied in five groups after application of progesterone (P), oestrogen (E), Progesterone and oestrogen (P + E) as luteal support hormones.

Materials/Methods: Female mice were superovulated by PMSG and HCG and then were mated with vasectomized mice; the mice were divided into two groups; experimental and control group. Experimental groups included three groups; hyperstimulated mice were given four consecutive daily

injections of progesterone, (P group), oestrogen (E group), oestrogen + progesterone (E + P group) and vehicle as a hyperstimulated control group. Natural control group mice were mated with vasectomized mice without any hyperstimulation. The uteri of all groups were collected after 4 days of hormone injection at window implantation phase. The uteri were prepared for histological and morphometrical studies.

Results: Morphological studies of endometrial tissue showed the luminal epithelium in P group was appeared cuboidal shape. The epithelium of E and E + P groups were seen in pseudostratified form. The epithelium of control and hyperstimulated groups were simple columnar. Morphometrical evaluation also showed height of the luminal epithelium in E and E + P were higher than those were seen in control and hyperstimulated groups. The height of epithelium in P group was the lowest in compared with other groups.

Conclusion: In an animal model of luteal phase support agents in hyperstimulated mice, exogenous administration hormones have effects on morphology of endometrial epithelium. Application of E + P increased the height of the epithelium and changed the morphology at the receptivity and implantation period of embryo. In addition, P alone cannot support luteal phase for requirement morphology compared with control group.

Infectious diseases and assisted reproduction

Altered ovarian function in women infected by chronic viral diseases undergoing IVF

Palmisano M, Lorusso F, Scioscia M, Serrati G, Bassi E, Totaro I, Vacca M, Masciandaro P, Depalo R

Department of Gynaecology, Obstetrics and Neonatology, Gynaecology and Obstetric Unit 'A'

Objective: A significant association between chronic viral diseases (CVD) and altered ovarian function have been described. The aim is to investigate the ovarian response and the clinical outcome of assisted reproductive techniques in females infected by CVD undergoing assisted reproduction.

Materials/Methods: Seven HIV (mean age, 36.7 ± 1.1 years), six HCV-HIV (37 ± 2), four HCV (34 ± 4) and six HBV (33.5 ± 4.4) were included in the study. All HIV patients were receiving antiretroviral therapy, while HCV and HBV patients had interrupted therapy with interferon at least one year before. A basal ovarian reserve screening was performed: antral follicle count (AFC), FSH and oestradiol levels were evaluated. GnRH agonist long protocols, with recombinant FSH (rFSH) were used for ovarian stimulation. The primary end-points were: dose of gonadotrophins, days of stimulation, oestradiol concentrations on human chorionic gonadotrophin day (HCG), total number of follicles and number of follicles ≥ 18 mm on HCG day and number of oocytes retrieved. The secondary end-points were: clinical pregnancy rate, defined as fetal cardiac activity on transvaginal sonography the ongoing pregnancy rate, defined as pregnancy progressing beyond week 12 of gestation.

Results: The groups were comparable for demographic characteristics. The amount of rFSH and the duration of stimulation were similar among the groups. On HCG day, significant differences were found in oestradiol concentrations ($P < 0.0001$), between HIV and HCV (403 ± 486 versus $1403 \pm$

1012 , $P < 0.05$), HIV and HBV (403 ± 486 versus 2613 ± 300 , $P < 0.001$) and HCV-HIV and HBV females (412 ± 229 versus 2613 ± 300 , $P < 0.001$), in the total number of follicles ($P = 0.0005$), between HIV and HCV (4 ± 2.3 versus 12.7 ± 5 , $P < 0.05$) and HIV and HBV females (4 ± 2.3 versus 14.3 ± 4.2 , $P < 0.001$), and in the mean number of follicles ≥ 18 mm ($P < 0.01$), between HIV and HCV (2 ± 1.6 versus 6.2 ± 2.5 , $P < 0.05$) and HBV patients (2 ± 1.6 versus 6.1 ± 3.2 , $P < 0.05$). The number of oocytes retrieved was significantly higher in HBV than in HIV (13 ± 6 versus 2.1 ± 2.7 , $P < 0.001$) and HCV-HIV patients (13 ± 6 versus 3 ± 2.5 , $P < 0.01$). The number of mature oocytes, fertilized and cleaved embryos were similar. Cancellation occurred in 57.2% of the HIV group, 16.7% in the HCV-HIV group and 50% in the HBV group. The pregnancy rate was 14.3% in HIV, 75% in HCV and 16.6% in HBV patients. The ongoing pregnancy rate was 14.3% in HIV, 50% in HCV and 16.6% in HBV patients per started cycle.

Conclusion: These results suggest that in patients infected with HIV or HCV-HIV, a severe ovarian dysfunction under the form of ovarian resistance to stimulation can be observed. This finding may be due to various endocrine perturbations linked to HIV status and to antiretroviral therapy. Both factors could have consequences on folliculogenesis and ovulation regulatory processes, leading to a reduced ovarian reserve and poor oocyte quality.

Impact of chronic viral diseases on semen parameters and success of viral removal after sperm washing

Lorusso F, Scioscia M, Vacca M, Masciandaro P, Palmisano M, Serrati G, Bassi E, Totaro I, Depalo R

Department of Gynaecology, Obstetrics and Neonatology, Gynaecology and Obstetric Unit 'A'

Objective: Infected patients can conceive safely, avoiding transmission risks to mother and children using sperm washing (SW) procedure in assisted reproductive techniques. Semen quality in infected males is essential to these procedures. However, the influence of the male's health status on semen parameters [human immunodeficiency virus (HIV), hepatitis C virus (HCV) and B virus (HBV)] is still debated. The aim of this study was to assess the impact of the virus infection on semen parameters and the ability of SW protocol to recover progressive motile spermatozoa.

Materials/Methods: Semen samples were obtained from 13 HIV (mean age, 38.8 ± 3.5 years), 17 HCV (mean age, 38.7 ± 2.7 years), 38 HCV-HIV (mean age, 39.4 ± 3.6 years) and 19 HBV (38.7 ± 6.2 years) infected males. All HIV patients were receiving antiretroviral therapy, whereas HCV patients had interrupted interferon at least 1 year before. All the samples were assessed as outlined by the World Health Organization. The semen samples were washed by a combined method: centrifugation on a discontinuous gradient, washing and migration by swim-up at 37°C and 5% CO_2 for 2 h. The motile spermatozoa that migrated to the top of the sperm medium were divided into two aliquots: one aliquot was used for insemination and the other for the presence of the virus using real-PCR. One-way ANOVA and Turkey multiple comparison tests were used for statistical analysis.

Results: The seminal volume and the sperm motility (a + b) were comparable among the four groups. The mean concentration (sperm/ml) was significantly different ($P = 0.0014$) among groups, with a statistically lower mean concentration in HBV (13.5 ± 12.6) and HCV males (14.4 ± 12), with respect to normal parameters, HIV (25.2 ± 14.6)

and HCV-HIV (24.7 ± 11) patients. The vitality was also significantly different among groups ($P = 0.0003$), with lower percentages in HBV patients (54.7 ± 31). HBV patients had also a lower rate of spermatozoa with normal morphology (21.6 ± 12 ; $P < 0.0001$) compared with the other groups (on average, 33.8 ± 8). The percentage of progressive motile spermatozoa recovered after SW in the different groups was significantly different ($P < 0.0007$), with a statistically lower percentage in HCV-HIV than in HCV (22.5 ± 14.5 versus 51 ± 37 , $P < 0.001$) and HBV males (22.5 ± 14.5 versus 42 ± 29 , $P < 0.05$). After sperm washing, the positive rate was 1.1%, attributable to one HCV sample that resulted positive.

Conclusion: In summary, HIV infection and antiretroviral treatment seem not to impair spermatogenesis in HIV positive males. In HCV infected men, the mean concentration is significantly reduced, whereas a significant impairment of seminal quality in terms of mean concentration, vitality and normal morphology is observed in HBV infected men. Sperm washing has been shown to be an effective procedure to obtain motile spermatozoa and to remove the virus from the semen.

Obtaining competent oocytes

Comparison of the morphology of oocytes at different cycles on the same patients: a different approach to examine the occurrence mechanisms of morphological abnormalities of oocytes

Aktas RG¹, Arat N¹, Ankarali H², Sofuoglu K¹, Cetinkaya T¹, Devranoglu B¹

¹ART Centre, Zeynep Kamil Women and Children Diseases Hospital, Istanbul; ²Department of Biostatistics, Faculty of Medicine, Karaelmas University, Zonguldak, Turkey

Objective: Oocyte quality affects early embryonic development, the pregnancy rate and fetal development. The mechanism(s) of morphological abnormalities on the oocytes are still unclear. Some studies support the hypothesis that these morphological changes reflect genetic characteristics of the patient, while others show the importance of stimulation protocols. There are also many reports demonstrating that the age of the patient is very important for the oocyte quality. However, which morphologic features of oocytes are affected from these facts are still unknown. The aims of this study were: (i) to clarify if certain morphologic features were always seen at the same patients; (ii) to examine if there are particular morphological features which were seen when patients got older; and (iii) to determine if specific morphological abnormalities were related with the stimulation protocol.

Materials/Methods: Eighty couples who underwent ICSI, with two or more cycles per couple, were included in this prospective randomized study. Abnormalities related with the oocyte morphology were classified as follows: (i) polar body anomalies; (ii) perivitelline space anomalies (wide/narrow perivitelline space, perivitelline debris, etc.); (iii) zona pellucida anomalies (thick/thin or irregular zona pellucida); (iv) cytoplasmic granulation (central or diffuse) (v) vacuolization in cytoplasm; (vi) oocyte membrane anomalies (easy penetration of ICSI pipet); (vii) smooth endoplasmic reticulum formation; (viii) refractile body formation; and (ix) shape anomalies of oocytes (small, giant or oval oocytes etc.). Records related to these morphological features of oocytes at each cycle were classified according to the age of the patient and the stimulation

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protocol. The following tests were used for statistical analysis: (i) *t*-test for proportional differences; (ii) paired *t*-test; and (iii) repeated measurement analysis of variance.

Results: Detailed results according to the each morphological anomaly after the statistical analysis were presented in the study.

Conclusion: This study gives some evidence on the occurrence mechanisms of some morphological changes on oocytes. More studies will be helpful to clarify this concern, and to obtain more oocytes of good quality.

Reactive oxygen species and total antioxidant in follicular fluid of women undergoing assisted reproductive techniques

Hassan HB¹, Esmail SM²

¹Department of Anatomy; ²Department of Biochemistry, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran

Objective: The role of free radicals and reactive oxygen species (ROS) in female reproductive function is still unclear. This study was designed to investigate the relationship between oxidative stress (MDA), total antioxidant capacity (TAC) and oestradiol concentrations in follicular fluid (FF).

Materials/Methods: Follicular aspirates obtained from women ($n = 10$) undergoing IVF following ovarian stimulation were evaluated using the ferric reducing antioxidant power (FRAP) assay for baseline total antioxidant capacity (TAC). Malondialdehyde concentrations were assayed by thiobarbituric acid reacting substances test. Total antioxidant capacity (TAC) and MDA concentrations were measured in plasma and FF spectrophotometric assays respectively.

Results: No significant correlation was observed in ROS concentrations in FF with embryo formation. There was no significant relationship between plasma and FF TAC. There was a significant positive association between FF oestradiol concentrations and TAC.

Conclusion: The positive association between FF oestradiol and total antioxidant capacity suggests that oestradiol may play a role in the ovarian antioxidant-oxidant balance. These results provide further evidence that ROS play a role in female reproductive function.

Effect of season on fertilization and embryo quality rates in assisted reproduction

Danfou M, Elmahaishi W, Elsrtaite O, Elmahaishi M

Faculty of Medicine, 7th October University and Misurata Infertility Centre, Misurata, Libya

Objective: Several studies have investigated seasonal variations during IVF as known causes for reducing the rate of success of in-vitro fertilization (IVF). Their results are contradictory, especially concerning fertilization and pregnancy rates. The aim of the present study was to re-evaluate these parameters using a large number of IVF cycles.

Materials/Methods: A total of 2250 ICSI cycles (first trial ICSI 1411) conducted in Misurata IVF Centre, Misurata, Libya between February 2002 and August 2006 were retrospectively analysed. To avoid bias in the evaluation of the fertilization rate, the second and third ICSI trials for some patients were considered for analysis. After adjusting for confounding by the age of the woman, type of infertility, indication for IVF and year of aspiration, some seasonal variation was observed in the oocyte maturation, oocyte quality, fertilization rate and embryo quality.

Results: There were statistically significant seasonal differences in Libya (Misurata) that influenced the outcome of IVF treatment. The highest fertilization and quality A embryo rates were observed during the Spring and the lowest in the Autumn. These changes correlated with the absolute number of light hours and its increment over time, but not with the temperature, humidity, or other environmental parameters.

Conclusion: Seasonality seems to have a significant influence on the fertilization process and on the quality of the human embryos that are obtained *in vitro*, possibly because of the light/dark variations over time. If this finding is confirmed, these seasonal changes should be taken into account when evaluating infertility data and in everyday clinical practice.

Expression of two survivin variants in different developmental stages of mouse ovaries

Mazoochi T¹, Salehnia M², Valojerdi MR², Mowla SJ²

¹Department of Histology, Kashan University of Medical Sciences, Kashan; ²Department of Anatomy; ³Department of Genetics, Tarbiat Modares University, Tehran, Iran

Objective: Survivin is one of the newer characterized members of the inhibitor of apoptosis proteins that shows widespread expression in embryonic and tumouric tissues. The aim of this study was to evaluate the expression of surviving mRNA variants in different stages of follicular development in mouse ovary.

Materials/Methods: This experimental study was carried out on immature female mice (NMRI). Preantral, large preantral and antral follicles mechanically isolated from ovaries in 14-, 18- and 21-day-old mice respectively. Total RNA was extracted from all above-mentioned groups and reverse transcribed by oligo dt primer and M-MLV enzyme. cDNA product was amplified by specific primer pairs for surviving and β_2 -m (as an internal control) during PCR. The semiquantitative expression of survivin mRNA in every stages of follicle was compared using post-hoc LSD test.

Results: The data showed expression of two survivin variants in different stages of follicles. Relative levels of surviving 40 and 141 did not change significantly in three stages of follicles. Surviving 121 mRNA was not expressed in all stages follicles.

Conclusion: Because of the similar relative abundance of two variant of survivin in different stage of follicles we concluded that other pro or antiapoptotic genes may be involved in follicular atresia.

This study was supported by Tarbiat Modares University, Tehran, Iran.

Comparison of the number of collected mature oocytes, age and the stimulation protocol at different cycles on the same patients

Aktas RG¹, Arat N¹, Ankarali H², Cetinkaya T¹, Devranoglu B¹, Sofuoglu K¹

¹ART Centre, Zeynep Kamil Women and Children Diseases Hospital, Istanbul; ²Department of Biostatistics, Faculty of Medicine, Karaelmas University, Zonguldak, Turkey

Objective: Patient's age and stimulation protocol affects oocyte quality and the number of matured oocytes. It was desired to examine: (i) how the number of matured oocytes changed after at least 6 months on the same patient; and (ii) if different stimulation protocols caused more matured oocytes at the same patient.

Materials/Methods: The study included 80 couples who underwent an ICSI programme, with two or more cycles per

couple. Spearman correlation analysis was used to examine the relationship between the number of mature oocytes and the age. Covariance analysis was used to compare the relationship between the stimulation protocols and the collected mature oocytes.

Results: There was a statistical meaningful relationship between the age and the matured oocytes in all cycles with the three different protocols ($r = -0.376$, $P = 0.001$ in first cycles of patients and $r = -0.362$, $P = 0.001$ in second cycles of the same patients). The decrease was especially evident when long protocol was used ($n = 52$; $r = -0.312$, $P = 0.024$). A statistically significant difference, regardless of age, was found between the short protocol and the antagonist protocol. Antagonist stimulation protocols resulted in the collection of more mature oocytes ($P = 0.015$). All results were compared with details in the study.

Conclusion: The study demonstrates statistically how the age and the type of stimulation protocol causes changes in the collection of matured oocytes.

Influence of ovarian stimulation regimen on the concentration of LIF, VEGF and nitric oxide concentration in serum and follicular fluid of IVF/ICSI

Hammadeh ME², Fischer-Hammadeh C², Micu R¹, Rosenbaum P², Schmidt W², Hatieganu I²

¹University of Medicine and Pharmacy, Cluj Napoca, Romania; ²Department of Obstetrics and Gynecology, University of Saarland, Homburg/Saar, Germany

Objective: The aims of this study were: (i) to evaluate the presence of LIF, VEGF and nitric oxide in serum and preovulatory follicular fluid (FF) in patients undergoing ovarian hyperstimulation for IVF/ICSI treatment; (ii) to determine the difference between the concentrations of these parameters in relation to ovarian stimulation regimens; and (iii) to find out the relationship between these parameters and ICSI outcome.

Materials/Methods: Seventy-five women undergoing IVF/ICSI therapy were included in this study. The women underwent ovarian stimulation with recombinant FSH (Gonal-F, G.1; $n = 40$), human menopausal gonadotrophin (HMG) (G.2, $n = 10$), or both FSH and HMG (G.3; $n = 25$) after pituitary downregulation with GnRHa. Serum and FF were collected at the time of oocyte retrieval and were frozen at -80°C until the measurement of LIF, VEGF and NO concentrations. LIF and VEGF concentrations were determined using enzyme-linked immunosorbent assay (ELISA), and NO was measured using the Griess reaction as an indirect assessment of NO activity.

Results: The mean age of the patients was similar in all three investigated groups (33.1 ± 4.2 ; 36.3 ± 5.4 and 34.6 ± 4.1 years respectively). The mean serum concentration of LIF, VEGF and NO in the G1 were $35.0 \pm 13.4 \mu\text{mol/l}$; $1.8 \pm 1.5 \text{ pg/ml}$ and $349.9 \pm 181.1 \text{ pg/ml}$ and ($39.4 \pm 8.0 \mu\text{mol/l}$, $44.9 \pm 20.4 \text{ pg/ml}$ and $3181.6 \pm 946.3 \text{ pg/ml}$ in follicular fluid. The corresponding values in G.2 were $42.2 \pm 22.5 \mu\text{mol/l}$; $1.7 \pm 1.1 \text{ pg/ml}$; and $410.8 \pm 135.4 \text{ pg/ml}$ in serum and $43.4 \pm 11.0 \mu\text{mol/l}$; $44.6 \pm 11.6 \text{ pg/ml}$ and $3182.3 \pm 1046.9 \text{ pg/ml}$ in follicular fluid. In the G.3, values were $373 \pm 20.0 \text{ mol/l}$; $2.4 \pm 2.6 \text{ pg/ml}$ and $388.0 \pm 182.4 \text{ pg/ml}$ in serum and the corresponding values in FF were $42.2 \pm 15.2 \mu\text{mol/l}$; $51.9 \pm 23.2 \text{ pg/ml}$ and $3749.3 \pm 620.0 \text{ pg/ml}$ respectively. A significant difference was found between VEGF in FF between G.1 and G.3 ($P = 0.042$). The mean number of retrieved, fertilized, transferred and the ongoing pregnancy rate in the G.1 was (6.9 ± 4.6 ; 4.3 ± 3.2 ; 2.0 ± 0.8 and 22.5%) and in G.2 were (5.9 ± 3.7 ; 3.2 ± 1.9 ; 1.8 ± 0.9 and 30% respectively).

The corresponding values in G. 3 were (5.3 ± 4.2 ; 3 ; 1 ± 3.0 ; 1.6 ± 0.9 and 28.0%). The mean number of retrieved fertilized, transferred oocytes and pregnancy rate were similar in all three investigated groups.

Conclusion: LIF and VEGF concentrations were significantly higher in FF in comparison with serum, which indicates that those cytokines are produced in the growing follicle and modulate hormone function in the ovary. However, their influence on oocytes and fertilization and implantation potential needs further investigation.

Follicular fluid VEGF concentrations and oocyte quality

Artini PG, Monteleone P, Simi G, Casarosa E, Cela V, Genazzani AR

Department of Reproductive Medicine and Child Development, University of Pisa

Objective: It has become increasingly clear that the follicular microenvironment of the maturing human oocyte is a determining factor for the implantation potential of an embryo deriving from that oocyte. Indeed, the quality and maturity of an oocyte are influenced by the level of intrafollicular oxygen content, which in turn is proportional to the degree of follicular vascularity. The aim of the study was to establish whether there is a relationship between follicular fluid VEGF concentrations, perifollicular vascularity and reproductive outcome in normal responders under the age of 35 undergoing IVF.

Materials/Methods: Sixty-one consecutive patients, all at their first IVF cycle, were included in the study. All patients had primary infertility due to male factor or tubal factor. At oocyte retrieval, the perifollicular vascularity of two follicles per ovary was estimated qualitatively through power Doppler blood flow, for a total of 244 follicles. The follicular fluid from the identified follicles was centrifuged and stored until VEGF assay. The maturity and fertilization rate of the corresponding oocytes as well as embryo quality and pregnancy rate were recorded.

Results: In our study, VEGF concentrations were significantly related to grade of perifollicular vascularity. Oocytes obtained from follicles with the higher grade of vascularization also showed a higher rate of fertilization, embryos, a better quality and higher pregnancy rates were obtained in women with highly vascularized follicles. Perifollicular blood flow Doppler indices seem to predict oocyte viability and quality. Moreover, VEGF may play a potential role in the development of the perifollicular capillary network.

Conclusion: The ability of a given follicle to express VEGF and develop an adequate vascular network may be inter-related in patients under the age of 35. An adequate blood supply may be fundamental important in the regulation of intrafollicular oxygen levels and the determination of oocyte quality

Genetic and epigenetic defects in germ cells

Mitochondrial DNA mutations and polymorphism in idiopathic asthenozoospermic men of Indian origin

Dada R¹, Kumar R², Venkat S², Shamsi MB², Kumar R³

¹Anatomy Department, AIIMS; ²AIIMS; ³Urology Department, AIIMS, North Delhi, India

Objective: Studies on sperm function, especially motility, turned attention to the possible role of sperm mitochondria in male infertility. There are no introns between genes but all

exons, so every change in mitochondrial DNA (mt DNA) is potentially lethal to cellular respiration. During spermatogenesis, spermatozoa require energy for biosynthetic processes and motility. Inhibition of sperm OXPHOS and rearrangements to the mitochondrial DNA genome can affect sperm function. As copy numbers of mitochondrial genome in spermatozoa are far fewer than somatic cells, slight damage to the mitochondrial genome results in impaired sperm function and infertility with less severe effect on other tissues and systems.

Materials/Methods: The whole mitochondrial genome was isolated from sperm and blood and mutations were screened with the help of DNA sequencing. The semen and blood samples analysed were obtained from 25 oligoasthenozoospermic idiopathic infertile men and 20 controls.

Results: G to A transition was detected in *ND4* gene at nucleotide position 11719 in sperm DNA of 19 cases and only 14 from blood DNA. Though this is a non-synonymous change, the amino acid composition remained the same. Polymorphisms in A750G, A4769G and A8860G have been found in all the semen as well blood DNA of the cases but only in 12 controls. A750G, A4769G are non-synonymous changes, but A8860G polymorphism in the *ATPase 6* gene changes amino acid threonine to alanine. Though A8860G is a known polymorphism in the Indian subcontinent, it should be looked at further, as its frequency seems to be greater in infertile men than in controls.

Conclusion: Further studies are in progress to see the effect of these mutations on the ATP depletion and reactive oxygen species (ROS) production.

Determination of Y chromosome microdeletions (AZF gene) in infertile men

Taga S¹, Leventerler H¹, Arıdoğan IA², Tuli A³, Ürünsak IF¹, Erçelen N⁴, Solmaz S⁵, Dikmen N³

¹Çukurova University, Faculty of Medicine, Gynecology and Obstetric Department-Centre of Assisted Reproduction; ²Urology Department; ³Biochemistry Department; ⁴Genetic Department of the VKV American Hospital; ⁵Histology and Embryology Department, Turkey

Objective: The frequency of Y chromosome microdeletions are determined in the Çukurova region and the importance of these microdeletions on spermatogenesis has been evaluated. Three different regions have been mapped on the long arm of the Y chromosome, named the 'azoospermic factor' (AZFa, b and c), are involved in the control of spermatogenesis. Microdeletions in these gene loci may result in azoospermia or severe oligozoospermia.

Materials/Methods: In this study, the frequency of Y chromosome microdeletions in 63 infertile males (38 azoospermic, 25 severe oligozoospermic) were evaluated by multiplex PCR (polymerase chain reaction) using 20 gene-specific primers (STS). Genomic DNA was purified from peripheral blood.

Results: Microdeletion frequencies were detected in all cases is 6.3 (4/63), 7.8 (3/38) of azoospermic, and 4% (1/25) of severe oligozoospermic group. One deletion was found in the azoospermic group, involving the complete AZFb and AZFc region and incomplete proximal c/d regions; other deletions in the azoospermic group involved AZFb and AZFc except the *DYS221* locus. One individual in the azoospermic group and one individual in the severe oligozoospermic group showed involvement only in the AZFc region.

Conclusion: We believe that, if the functions of all the genes that play a role in spermatogenesis are explained, it will be important to the understanding of the aetiology of male infertility.

Outcome of assisted reproduction procedures

Intracytoplasmic sperm injection is not justified for poor responders

Poli M, Keane D, Mocanu E

Human Assisted Reproduction Ireland, Rotunda Hospital, Dublin, Ireland

Objective: There is a growing body of evidence that ICSI should be used only in specific cases of male factor infertility. Yet it is still a common belief that ICSI could give a better chance of fertilization, cleavage and general development to an embryo when compared with IVF. We set out to establish if ICSI is better than IVF in cases of poor ovarian responders (where three or fewer oocytes are collected).

Materials/Methods: This is an 8-year retrospective, electronic database and single chart review of all assisted reproduction cycles undertaken in a tertiary referral/academic unit in Ireland. All cycles with three or fewer oocytes retrieved were included in the study. The study population ($n = 419$) was divided according to the laboratory procedure performed (IVF: group 1 or ICSI: group 2). The following parameters were compared: transfer, fertilization and cleavage rates; positive pregnancy test, number of pregnancy sacs and fetal hearts per cycle started.

Results: In the two groups analysed, fertilization (66.6 versus 67.5%) and cleavage rates (89.4 versus 87.3%) were comparable. The transfer rate and mean number of embryos transferred were also similar, (70.2 versus 68.8%) and (1.55 versus 1.6) respectively. No significant differences were present in both implantation rates and clinical pregnancy rates per cycle started, when comparing group 1 with group 2 (12.8 versus 8.2% and 11.3 versus 7.0%).

Conclusion: This study shows that in the context of poor ovarian response (three or fewer oocytes collected), ICSI does not offer a better chance of pregnancy. While not statistically significant, clinical pregnancy rates per transfer were notably higher in group 1 when compared with group 2 (16.1 versus 10.1%). This demonstrates that IVF should be the procedure of choice rather than ICSI in this highly selected group of patients. These findings suggest that a prospective randomized trial should be performed to further assess these results.

IVF productivity measures using NPSU public data

Stanger J¹, Yovich J²

¹FertAid Pty Ltd; ²PIVET Medical Centre

Objective: Public information available from the Australian National Perinatal Statistics Unit (NPSU) was used to develop national productivity measures for embryology laboratory.

Materials/Methods: NPSU reports between 1993 and 2005 were accessed. Published data on the number of egg collections (IVF, ICSI and GIFT) and transfers, the number of oocytes collected (2002–2005 only), the number of viable embryos (embryos of suitable quality to transfer or freeze), the number of embryos transferred (fresh or frozen) and the number of babies (fresh and frozen) were either extracted or calculated from tables within the each report.

Results: Between 1993 and 2005, the number of egg collections rose from 12,050 to 27,995 and the number of babies delivered rose from 2539 to 9395. The total number of embryos transferred both fresh and frozen also rose from 39,627 to 51,463 in 2003 and falling to an estimated 47,014 in 2005. The average number

transferred per OPU (fresh and frozen) fell from 3.2 in 1994 to 1.7 in 2005. Between 65 and 80% of all viable embryos have been transferred. Of more importance, the total number of viable embryos (transferred or frozen) rose from 48,608 to 78,049 over this period, but per OPU was static at between 2.7 and 4.5. The number of babies per OPU rose from 20/100 OPU to 38/100 OPU mirrored the number of babies/100 viable embryos that increased from 6 to 12. Estimates of babies per oocyte collected were between 3.4 and 4.4%.

Conclusion: Analysis of Australian IVF productivity over a 15-year period where the contribution of fresh and frozen transfers were combined irrespective of age and other distracting factors provide a unique insight into the productivity of the industry. Benchmark indicators for the industry may allow individual clinics to review their performance over all patients and treatment options. Embryo quality performance indicators further suggest that few viable embryos may lead to term conceptions and that other indicators of viability may be required. This information may be valuable when considering other treatments such as oocytes cryopreservation. The data also suggest that few embryos of a quality suitable for transfer of freezing ultimately have the potential for pregnancy and that a large number of oocytes are required to generate a single baby.

PGD/PGS

Preimplantation genetic testing outcomes for poor prognosis couples carrying cryptic translocations

Ercelen N¹, Tutar E¹, Gultomruk M¹, Balaban B², Alatas C², Yakin K², Urman B²

¹VKV American Hospital Genetic and Genomic Sciences Centre; ²VKV American Hospital Women's Health Centre, IVF Department, Istanbul, Turkey

Objective: To present the results of preimplantation genetic testing for translocations in 18 cycles.

Materials/Methods: Cytogenetic analysis was performed on cultured lymphocytes derived from the peripheral blood of couples. Two telomeric probes with two or one centromeric probes for the chromosomes involved in the translocation were analysed on metaphase spreads of the translocation carrier individual. Following biopsy of a blastomere from day-3 embryos, they were analysed for five chromosomes (13, 16, 18, 21, 22), with PB probe (Vysis Inc.), which are common in aneuploidy. Abnormal embryos were not further analysed. The remaining nuclei were then analysed with telomeric and centromeric probe sets specific for each cycle. Embryos available for transfer were transferred on day 5 of embryo development. Amniocentesis was recommended for pregnant patients.

Results: Preimplantation genetic testing was performed for 18 translocation carrier cycles, with an average maternal age of 31.6 years, involving 112 embryos, of which only 14.3% (16/112) were normal or balanced. A total of 14.7% (11/75) of embryos were analysed normal or balanced in embryos of reciprocal translocations, while 13.5% (5/37) of embryos were analysed normal in those of Robertsonian translocations. An average of 1.6 (16/10) normal embryos were transferred for 10 cycles and three clinical pregnancies resulted with a 28.6% (2/7) and 33.3% (1/3) pregnancy rate per embryo transfer in reciprocal and Robertsonian translocations, respectively.

Conclusion: Preimplantation genetic testing should be advised for poor prognosis translocation carrier couples to reduce spontaneous abortions and to have successful pregnancy. Of

the 18 cycles, we had translocation carrier couples in whom translocation originated from the mother (7 cycles) or the father (11 cycles). Out of 112 biopsied embryos, only 16 embryos were analysed as normal or balanced.

Should preimplantation genetic diagnosis be indicated in advance maternal age?

Milan M, Rubio C, Pehlivan T, Rodrigo L, Mateu E, Vilorio T, Peinado V, Mercader A, Buendia P, Delgado A, Bronet F, Remohi J, Pellicer A
Institut Universitari IVI, Valencia, Spain

Objective: To date, few prospective randomized controlled trials about preimplantation genetic diagnosis (PGS) in advanced maternal age patients (AMA) have been published. These studies have shown either similar or worse outcome results in the PGS groups when compared with controls. This is an important message for those practising reproductive medicine; the embryos may have been overmanipulated in IVF laboratories without scientific justification. However, all of these papers have major drawbacks, such as low sample size, high percentage of undiagnosed embryos (20%) and lack of important chromosomes in the analysis, such as chromosomes 15 and 22.

Materials/Methods: We decided to retrospectively analyse our database from 2001 to 2006. Only 476 PGS cycles were included (women >37 years) as the result of a very strict selection process, excluding other potential indications of PGS, such as implantation failure or recurrent miscarriage. The outcome of these cycles was compared with 1630 cycles of IVF without PGS in patients of the same age.

Results: Ongoing implantation rates (OIR) were significantly ($P < 0.0001$) different when PGS (21.4%) was compared with conventional IVF (7.4%). Overall pregnancy rates per transfer were not affected (24.3% PGS versus 26.0% IVF, $P > 0.05$), but the number of embryos replaced in PGS was 1.4 ± 0.5 versus 2.5 ± 1.1 in IVF. Moreover, we cytogenetically analysed some of the miscarriages in an attempt to learn about the chromosomal contribution to these unsuccessful results. In patients without PGS, 10 out of 14 miscarriages showed chromosomal abnormalities, most of them for chromosomes included in our PGS panel (13, 15, 16, 18, 21, 22, XY). In the PGS group, seven specimens from aborted material were studied and all of them showed anomalies for chromosomes not included in our FISH panel.

Conclusion: This study demonstrates that if PGS were applied in the IVF cycle, probably many miscarriages could be avoided. Moreover, two pregnancies achieved without PGS were terminated due to trisomy 18 and 21. We truly believe that improvements in the analysis of the entire set of chromosomes would certainly increase our ability to choose the right embryos, which would lead to ongoing and healthy pregnancies.

Cumulative experience with preimplantation genetic screening in implantation failure patients

Budak TP, Rubio C, Rodrigo L, Mateu E, Mercader A, Buendia P, Vilorio T, Remohi J, Simon C, Pellicer A
Instituto Valenciano De Infertilidad, University of Valencia, Spain

Objective: Repeated implantation failure (IF) is usually defined as three or more failed embryo transfer procedures. PGS (preimplantation genetic screening) is one of the treatment strategies offered at the IVF setting in this group of patients. There are reports of the benefit of PGS in this poor patient group.

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Materials/Methods: Here we present our cumulative experience between the period of 1/1/2000 to 31/07/2007 in a total of 454 PGS cycles in patients with ≥ 3 previous implantation failures. Patients underwent a PGS cycle with day-3 embryo biopsy and analysis of blastomeres with fluorescence in-situ hybridization (FISH) for chromosomes 13, 15, 16, 18, 21, 22, X and Y. Chromosomally and morphologically normal embryos were transferred on day 5.

Results: Overall the implantation rate was 29.3%, the pregnancy rate was 37.4% and, clinical abortion rate was 19, 8%. In patients with ≤ 37 years of age the implantation rate was 30.3%, the pregnancy rate was 39.1% and, clinical abortion rate was 14.4%. In patients >37 years of age the implantation rate was 26.7%, the pregnancy rate was 33.6% and clinical abortion rate was 33.3%. When results were evaluated in patients with at least one blastocyst transferred, the results were as follows: in patients with ≤ 37 years of age, the implantation rate was 33.8%, the pregnancy rate was 45.1% and, clinical abortion rate was 15.7%. In patients with >37 years of age, the implantation rate was 29.6%, the pregnancy rate was 38.1% and, clinical abortion rate was 31.3%. When the miscarriages observed in these patients were analysed depending on oestradiol concentrations, in patients ≤ 37 years of age in whom 13 miscarriages were observed, five of them were in patients with oestradiol concentrations of >2500 pg/ml. In patients >37 years of age in whom 12 miscarriages were observed, six were patients with oestradiol concentrations of >2500 pg/ml. These results show that the reason for the miscarriages could be due to high oestradiol levels or endometrial factors.

Conclusion: Acceptable pregnancy and miscarriage rates are obtained with PGS in this poor prognosis patient group.

Identification of good prognostic factors for preimplantation genetic diagnosis in unexplained recurrent miscarriage couples

Buendía P, Rubio C, Mercader A, Rodrigo L, Mateu E, Pehlivan T, Vilorio T, Peinado V, Delgado A, Milán M, Gadea B, Simón C, Remohí J, Pellicer A
Instituto Universitario IVI, Valencia, Spain

Objective: Chromosome abnormalities, arising *de novo* from random errors produced during gametogenesis and embryonic development, may be an important aetiological factor in some patients with unexplained recurrent miscarriage (RM). The objective of our study was to identify specific subgroups of RM patients in whom the selection of chromosomally normal embryos for transfer would improve their reproductive outcome, by decreasing the risk of a further miscarriage.

Materials/Methods: Retrospective study of 404 cycles, from patients with normal karyotypes and two or more previous abortions of unknown aetiology included in our preimplantation genetic diagnosis (PGD) programme (January 1997 to July 2007). The incidence of chromosomal abnormalities, pregnancy, implantation and miscarriage rates were evaluated according to the following factors: women's age, miscarriages from spontaneous pregnancies or from fertility treatments, presence of chromosomal abnormalities in a previous miscarriage/pregnancy, number of previous miscarriages and presence of chromosomal abnormalities in sperm samples. Embryo biopsy was performed on day 3 and blastomeres were analysed by fluorescence in-situ hybridization (FISH) for chromosomes 13, 15, 16, 18, 21, 22, X, and Y. Chromosomally normal embryos were transferred on day 5. Statistical comparisons were performed using a Fisher's exact test ($P < 0.05$).

Results: Overall pregnancy, implantation and miscarriage rates were 40.3, 31.5 and 18.2% respectively. In patients ≤ 37 years of age, we observed significantly higher ongoing pregnancy (40.7 versus 20.2%, $P < 0.001$), ongoing implantation (30.6 versus 13.3%, $P < 0.001$) and, miscarriage (10.9 versus 28.6%, $P < 0.05$) rates compared with patients over 37 years of age. The incidence of chromosomal abnormalities was higher in patients > 37 years (62.8 versus 72.0%, $P < 0.001$).

In patients ≤ 37 years results were compared taking into account different factors: chromosomal abnormalities in previous miscarriages/pregnancies: significantly higher incidence of embryos with chromosomal abnormalities (73.9 versus 63.2%, $P < 0.01$), ongoing pregnancy rate (72.2 versus 56.7%, $P < 0.01$) and ongoing implantation rate (43.7 versus 33.5%, $P < 0.01$) was observed in couples with a previous aneuploid miscarriage. Despite not being significant, no miscarriages were observed when PGD was performed in RM couples with previous miscarriages from fertility treatments compared with couples whose miscarriages were from spontaneous pregnancies (12.7%). Although not significant, couples with abnormal FISH on spermatozoa did not miscarry after a PGD cycle, whereas couples with normal FISH results in spermatozoa showed 16.7% miscarriage rate. A significantly lower ongoing implantation rate (9.1%) was observed in couples with ≥ 5 previous miscarriages, associated to a lower percentage of chromosomally abnormal embryos.

Conclusion: We can conclude that PGD would be strongly recommended when RM is associated with miscarriages from infertility treatments, previous chromosomopathy, fewer than five previous miscarriages and, increased incidence of chromosomal abnormalities in spermatozoa.

Outcomes of preimplantation genetic diagnosis

Ercelen N¹, Comert H¹, Erkan L¹, Ilbay O¹, Mercan R², Balaban B², Isiklar A², Urman B²

¹American Hospital Genetics and Genomic Sciences Centre;

²American Hospital IVF Centre, Istanbul, Turkey

Objective: To present the results of preimplantation genetic diagnosis (PGD) studies for the identification of a causative gene mutations together with/without human leukocyte antigen (HLA) typing in several single gene disorders.

Materials/Methods: Following standard IVF protocol, single embryonic cells were removed from day-3 embryos following oocyte retrieval. Negative control for each blastomere was also prepared to control the contamination risk. Multiplex semi-nested PCR by using specific primers and polymorphic markers specific for each disease was applied for the mutation analysis. To detect and avoid misdiagnosis due to allele drop-out (ADO), a haplotype analysis for father, mother and affected child was performed for each family before the preimplantation HLA typing. HLA genes from blastomeres are tested together with short tandem repeats (STR) in the HLA regions by using multiplex semi-nested PCR system.

Results: In total, 206 embryos were tested for different single gene disorders in 27 PGD cycles performed for 21 couples (7.6 per cycle). 49 embryos (23.8%) were selected and transferred (approximately 1.8 per cycle). Seventy-eight embryos from eight patients were analysed for specific gene mutation and HLA compatibility. Thirteen unaffected embryos, which were HLA-matched to their affected siblings, were selected and transferred. Two clinical pregnancies were obtained from the PGD for HLA typing. One was twin and the other was singleton. Twins and

singleton newborns are potential donors of stem cells for their affected siblings. Six out of obtained seven clinical pregnancies resulted in nine healthy live-births (with the implantation rate of 20.4%), and one twin pregnancy is still ongoing.

Conclusion: These outcome data showed that due to its accuracy and efficiency, application of single-cell PCR based DNA analysis is a useful tool for the PGD of monogenic disorders. In addition, HLA typing during PGD not only offers production of unaffected offspring, but also possible treatment of affected siblings.

Follicle culture and oocyte maturation

MCAK is involved in silencing of the spindle checkpoint in mammalian oocytes

Vogt E, Sanhaji M, Kipp A, Eichenlaub-Ritter U

University of Bielefeld, Faculty of Biology, Gene Technology/Microbiology, Bielefeld, Germany

Introduction: Altered gene expression affecting motor proteins and cell cycle regulators causing permissive checkpoint control and spindle formation may contribute to high susceptibility of human oocytes to errors in chromosome segregation (Hamatani *et al.*, 2004; Steuerwald *et al.*, 2007; Vogt *et al.*, 2008). We studied the distribution and role of MCAK (mitotic centromere associated kinase, KIF2C) in mammalian oocytes, a microtubule depolymerase belonging to the kinesin-like family of microtubule motor proteins (Kin I kinesins) with internally located catalytic motor domain (Wordemann *et al.*, 2007). Since Aurora B kinase (AURKB) phosphorylates and deactivates MCAK in somatic cells (Wordemann *et al.*, 2007), mouse oocytes were matured in presence of AURKB inhibitor to maintain constitutive MCAK activity.

Materials and methods: Maturation, spindle formation, chromosome congression and distribution of MCAK was analysed after maturation without and with AURKB inhibitor (1.5 mM ZM447439) or knockdown of MCAK or Mad2 expression by RNAi methodology. Oocytes were stained by anti-tubulin, anti-Mad2 and -Bub1R checkpoint proteins (Eichenlaub-Ritter *et al.*, 2007), or anti-MCAK (Wordemann *et al.*, 2007) or spread and stained by C-banding (Eichenlaub-Ritter *et al.*, 2007).

Results: MCAK is recruited to chromosome arms after GVBD later associates with outer kinetochores at prometaphase I and at metaphase II, and localises at inner centromeres at late metaphase I and anaphase I. Knockdown of MCAK by RNAi does not inhibit bipolar spindle assembly and congression of chromosomes after extended meiosis I arrest, but inhibits progression to anaphase I. Blocked GVBD oocytes possess bivalent chromosomes. Double knockdown of expression of MCAK and the spindle checkpoint component Mad2 results in anaphase I progression. However, the MCAK and Mad2 depleted metaphase II oocytes have highly aberrant spindles and unordered chromosomes. Maturation in AURKB inhibitor induces meiotic arrest and formation of aberrant MI spindles with unaligned chromosomes. Bub1R checkpoint protein is expressed at centromeres indicative of prolonged activation of the spindle assembly checkpoint (SAC) in presence of constitutively active MCAK but checkpoint control appears leaky. Some of the blocked GVBD oocytes are polyploid while others contain several or single metaphase II chromosomes together with bivalents.

Conclusion: MCAK is an integral component of centromeres of meiotic chromosomes in oocytes and appears necessary to silence the SAC in mammalian oocytes. By contrast, inhibition of AURKB-mediated phosphorylation of MCAK, probably inducing constitutively active MCAK and interfering with other regulatory processes interferes with cytokinesis and coordinated separation of chromosomes at anaphase I as well as normal chromosome congression at meiosis II. Disturbances in abundance and activity of MCAK or AURKB can therefore critically affect meiotic progression, spindle formation and timely separation of chromosomes in mammalian oocytes and thus predispose to meiotic errors. This may be particularly relevant for aged oocytes.

Effects of different concentrations of selenium on the in-vitro maturation of preantral follicles in serum-free and serum supplemented culture systems

Elahi AA¹, Salehnia M¹, Allameh AA²

¹Anatomy Department, Faculty of Medical Sciences;

²Biochemistry Department, Faculty of Medical Sciences, Tarbiat Modares University Tehran, Iran

Objective: Selenium is an essential trace element in conventional tissue culture media to guarantee adequate biosynthesis of selenoprotein in cellular antioxidant system to protect the cells from oxidative damage. This study investigated the effect of sodium selenite (SS), on developmental ability and quality of in-vitro maturation of mouse preantral follicles.

Materials/Methods: The preantral follicles were mechanically isolated from ovaries of 12- to 14-day-old NMRI mice and cultured in TCM serum free medium in the different concentrations (0, 5, 10, 15 ng/ml) of SS. The medium supplemented with 3 mg/ml bovine serum albumin (BSA) or 5% fetal bovine serum (FBS) in 5% CO₂ in air at 37°C for 12 days. At day 12 of culture ovulation was induced by addition of 1.5 IU/ml human chorionic gonadotrophin. Released oocytes were classified as germinal vesicle (GV), metaphase I (MI) and metaphase II (MII).

Results: The survival rate of follicles cultured in BSA supplemented groups containing 0, 5, 10, 15 ng/ml SS were 69.37, 73.74, 74.10 and 76.45% respectively and also that of follicles cultured in FBS supplemented groups with the same concentrations of SS were 76.53, 88.23, 90.83 and 78.93% respectively. The diameters of preantral follicles cultured in different concentrations of SS in BSA groups were 169.33 ± 14.56, 179.18 ± 19.77, 187.07 ± 20.51, 189.22 ± 20.95 and in FBS supplemented groups were 170.16 ± 17.97, 182.79 ± 18.72, 199.84 ± 15.58, 188.23 ± 21.54 respectively. There were significant differences between the survival rate and follicular diameter on day 4 of culturing in 10 ng/ml SS in FBS supplemented group with others. The percentage of MII oocyte (33.08%) was significantly higher in the 10 ng/ml SS group.

Conclusion: These results demonstrated that the medium containing 10 ng/ml SS in FBS supplemented culture system improve the in-vitro growth maturation of mouse preantral follicles.

Ultrastructure of isolated preantral follicles co-cultured with cumulus cells in the presence and absence of leukaemia inhibitory factor

Salehnia M, Haidari K, Valuredi M

Anatomy Department, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Objective: This study was designed to verify the ultrastructural characteristics of in-vitro-cultured mouse preantral isolated follicles in co-culture system in the presence and absence of leukaemia inhibitory factor (LIF).

Materials/Methods: Mechanically isolated preantral follicles were co-cultured with cumulus cell in α -minimal essential medium (α -MEM) supplemented with 5% fetal bovine serum, 100 mIU/ml recombinant FSH, 1% ITS (insulin, transferrin and selenium), 20 ng/ml murine recombinant epidermal growth factor and 50 ng/ml LIF for 4 days. The follicles were processed and sectioned for transmission electron microscopy examination.

Results: The oocyte and granulosa cells showed more maturation features in the co-cultured system than in the simple one. In co-cultured follicles, the aggregation of mitochondria around the germinal vesicle was prominent and the granulosa cells contained mitochondria with tubular cristae, a well developed smooth endoplasmic reticulum and several large lipid droplets.

Conclusion: The co-culturing and LIF supplementation could be effective technique to improve the development of isolated preantral follicles *in vitro*.

Survival rate of preantral follicles derived from vitrified neonate mouse ovarian tissue by conventional method

Safari S¹, Eimani H^{2,3}, Mehranjani MS¹, Abnosi MH¹,

Valojerdi MR², Yazdi PE²

¹Department of Biology, Faculty of Basic Science, Arak University; ²Department of Embryology, Royan Institute, Tehran; ³Department of Anatomy, Faculty of Medicine, Baqiyatallah University, Tehran, Iran

Objective: Cryopreservation of ovarian tissue has generally been performed using slow-freezing methods, but using of vitrification (a rapid and simple cryopreservation method) has been shown to be effective for cryopreservation of ovarian tissues. The aim of this study was to investigate the survival rate of preantral follicles isolated from vitrified ovarian tissue by conventional methods.

Materials/Methods: Ovaries of 14-day-old mice were separated and divided into three groups: group I: vitrified by conventional straw; group II: treated with equilibration and vitrification solutions and followed by thawing process without immersion in liquid nitrogen; control group not treated by equilibration-vitrification media and vitrification. Ovaries in group I were immersed in equilibration and vitrification solution composed of ethylene glycol, dimethylsulphoxide in HEPES-buffered tissue culture medium 199 with human serum albumin and loaded into conventional straws, then immediately plunged into liquid nitrogen. After 3 weeks the vitrified ovaries were warmed. Preantral follicles from ovaries in three groups were isolated by mechanical dissection. Good-quality isolated follicles were cultured for 4 days to compare growth and survival rate between mentioned groups.

Results: Survival rates in group II (97.3%), as in the control group (98.7%), were significantly higher ($P < 0.0001$) than in group I (47.7%). Increasing in follicles diameter after 4 days in group I was significantly lower ($P < 0.001$) than the control and group II.

Conclusion: Results suggest that conventional vitrification of mouse ovarian tissue is not an appropriate method for preantral follicles preservation. The cause of reduced survival rate of preantral follicles in this method, can be the indirect contact of ovaries with liquid nitrogen.

Effect of erythropoietin on bovine in-vitro embryo production

Conde P^{1,2}, Herrera C², Gutierrez G^{1,2}, Quintans C², Gentile T¹, Pasqualini S²

¹Instituto de Estudio de la Inmunidad Humoral 'Dr Ricardo Margni'. CONICET, Facultad de Farmacia y Bioquímica, UBA, Junín 956; ²Halitus Biotecnología, Halitus Instituto Médico, M. T. De Alvear 2084, C1112AAF, Buenos Aires, Argentina

Objective: Erythropoietin (EPO) is known to regulate the number of circulating red blood cells. Recently, EPO and EPO mRNA were found in organs non-related to erythropoiesis, such as testicle, endometrium, oviduct and ovary. The aim of our work was: (i) to evaluate the effect of adding EPO to the in-vitro maturation medium (IVMm) of bovine oocytes on the percentage of MII oocytes, cleavage and blastocyst rates as well as blastocyst cryotolerance; and (ii) to evaluate the effect of adding EPO to the IVF medium on fertilization rates.

Materials/Methods: Bovine oocytes were obtained from slaughterhouse ovaries and for experiment 1 they were matured in vitro for 22 h in groups of 5–10 in 50 µl droplets of IVMm (De Matos et al., 2002) with 0, 7, 14, 20 or 80 mIU/ml EPO. Then, they were denuded from their cumulus cells or incubated for 24 h with 2×10^6 bull sperm/ml. Presumptive zygotes were denuded and cultured for 24 h in synthetic oviduct fluid medium (SOFm) in coculture with granulosa cells and then the medium was replaced for SOFm with 1.5 mmol/l glucose. The embryos remained in this medium for 7 additional days. Blastocysts obtained were cryopreserved in 0.25 ml straws, with a slow freezing curve (0.6°C/min). After that, they were plunged into liquid nitrogen. Thawing was performed by keeping straws at room temperature for 10 s, followed by immersion in a water bath at 36°C and culture in SOFm with 1.5 mmol/l glucose for 48 h. The maturation, cleavage and blastocyst rates were compared between the different groups. In experiment 2, the oocytes were matured as in experiment 1, without EPO, and incubated with the same concentration of spermatozoa with 0, 8, 40 mIU/ml EPO. Cleavage and pronuclear formation rates were compared between groups. Differences among groups were analysed by chi-squared test.

Results: The supplementation with EPO during IVM demonstrated a positive effect only on the 14 mIU/ml group by a significant increment on the thawed hatched blastocyst rate (71 versus 39% of the control group, $P < 0.05$). No significant differences were obtained when EPO was added to IVF.

Conclusion: These results demonstrate an improvement on the efficiency of bovine in-vitro embryo production (IVP). To our knowledge, this is the first report on the effect of EPO on in-vitro embryo development. Further investigations will be necessary to determine the effect of adding EPO during embryo development as well as its regulation in reproductive organs.

Sperm function

Quality of testicular tissue does not influence the outcome of in-vitro fertilization treatments

Fancsovsits P¹, Rusz A², Gilán ZT¹, Murber A¹, Horváth LZ¹, Hauzman EE¹, Romics P², Rigó J Jr¹, Urbancsek J¹

¹First Department of Obstetrics and Gynaecology, Semmelweis University School of Medicine, Budapest, Hungary; ²Department of Urology, Semmelweis University School of Medicine, Budapest, Hungary

Objective: Poor quality of testicular tissue may pose unsolvable difficulties in some cases of TESE-ICSI treatment. Biopsies containing very low sperm numbers result in technical difficulties during processing the testicular tissue. Searching sperm in these tissues is time consuming and it is not guaranteed that sufficient number of spermatozoa can be collected. The aim of this study is to analyse the effect of the quality of testicular tissue on the outcome of TESE-ICSI treatments.

Materials/Methods: ICSI treatments with spermatozoa obtained from frozen-thawed testicular tissue were performed in the First Department of Obstetrics and Gynaecology in collaboration with the Department of Urology, Semmelweis University, Budapest, between January 2001 and August 2007. Data of 106 TESE-ICSI treatments of 49 patients were analysed. A total of 853 mature oocytes were injected with testicular spermatozoa. On the basis of microscopic analysis with 200-fold magnification, testicular tissue was classified into the following groups: I: >5 spermatozoa per microscopic view; II: 1–5 spermatozoa per microscopic view; III: <1 spermatozoon per microscopic view (occasionally). TESE-ICSI cycles of these categories were compared with regard to fertilization rate, embryo development, and pregnancy rate. Chi-squared and Kruskal-Wallis ANOVA tests were used for statistical analysis.

Results: We did not find significant difference between groups with regard to maternal age, length of hormonal stimulation, numbers of oocytes or collected mature oocytes. Fertilization rates were similar among groups [I: 51.6%; II: 48.0%; III: 47.3%; ($P = 0.57$)]. Cell number and quality of transferred embryos did not differ significantly between groups. We failed to find any significant difference between groups in pregnancy rates [I: 22.1%; II: 30.0%; III: 27.8% ($P = 0.72$)].

Conclusion: Our results show that number of spermatozoa found in testicular tissue has no influence on the outcome of TESE-ICSI treatments. Accordingly, we can expect an acceptable chance for achieving a pregnancy even in cases of very poor quality testicular tissue.

Effect of calcium, magnesium, zinc and copper in blood and seminal plasma on semen parameters of spinal cord injured men compared with normal controls

Salsabili N¹, Mehraei A², Jalaie S³

¹IVF Department, Mirza Kouchak Khan Hospital; ²Urology Research Centre; ³Rehabilitation Faculty, Tehran University of Medical Sciences, Tehran, Iran

Objective: The aim of the study was to determine and compare the effect of calcium (Ca), magnesium (Mg), zinc (Zn) and copper (Cu) concentration in blood and seminal plasma on sperm parameters of spinal cord injured (SCI) men and normal controls.

Materials/Methods: A prospective study was designed. In total 93 SCI men and 145 neurologically intact (NI) men provided a standardized blood and semen specimen. Total Ca and Mg concentrations were determined with colorimetric and point assay procedures. Zn and Cu were determined by flame atomic absorption spectrophotometer. Semen analysis was performed according to the World Health Organization (WHO, 1999).

Results: The sperm parameters in the SCI group were significantly lower than in the NI group; in motility 23.19%, in normal morphology 14.5%, and in viability 41.51%. The number of leukocytes, at $15.4 (\pm 17.82) \times 10^6/\text{ml}$, was higher than the NI group at $5.26 (\pm 9.03) \times 10^6/\text{ml}$. In SCI, the mean concentrations of seminal Zn and Cu (126.3 ± 9.7 , $146.05 \pm$

52.88) $\mu\text{g/dl}$ was higher than the blood plasma Zn and Cu (112.27 ± 31.64 , 123.73 ± 27.88) $\mu\text{g/dl}$ ($P < 0.05$) and plasma Zn was related to the number of spermatozoa ($r = 0.285$; $P < 0.001$), whereas seminal Zn had a negative correlation with the number of spermatozoa ($r = -0.213$; $P < 0.04$) and a positive correlation with sperm motility ($r = 0.311$; $P < 0.002$). Plasma Cu had a negative correlation with sperm motility ($r = -0.219$; $P < 0.03$) and linear correlation with the number of leukocytes ($\text{Cu} = 113.379 + 1.078 \times \text{no. of leukocytes}$) in seminal plasma, but seminal Cu had a positive correlation with the sperm motility ($r = 0.214$; $P < 0.03$). In the NI group, seminal Zn and Cu (134.02 ± 70.86 , 171.15 ± 68.48 $\mu\text{g/dl}$) were higher than the blood plasma Zn and Cu (98.90 ± 14.68 , 112.27 ± 29.28 $\mu\text{g/dl}$) ($P < 0.001$).

Conclusion: Ca, Mg, Zn, and Cu play an essential role in spermatogenesis and fertility, especially in the motility of spermatozoa. The low amount of seminal Zn, Cu in the SCI group may be attributed to urogenital tract infection and prostatitis, which was common in this group. The lack of association between the seminal elements in its ionized form with sperm parameters indicates that the determination of these elements in blood and seminal plasma does not discriminate fertility in spinal cord injured men. Therefore, the routine determination of these elements cannot be recommended during basic infertility investigation.

Comparison between subjective and objective assessment of human sperm morphology: computer-assisted morphometric evaluation has a better repeatability

Crausaz M, Parapanov R, Senn A, Germond M
FABER Foundation, Rue de la Vigie 5 CH-1007 Lausanne, Switzerland

Objective: Subjective analysis of human sperm morphology is strongly dependent on the technician's experience, which induces inter-laboratory variations. Consequently, the clinical interpretation of the results is often difficult. Computer-assisted sperm analysers (CASA) provide a mean to standardize sperm morphology. Using spermatozoa obtained from young and healthy volunteers, the performance and repeatability of the classical subjective approach was compared with that of a CASA system.

Materials/Methods: The studied population consisted of 795 young volunteers (19.9 ± 1.3 year old) attending military recruitment in Switzerland. These men were included in a larger study on the effects of endocrine disruptors on the environment and on human reproduction. Thin smears were prepared using 5–10 μl native semen, air-dried and Papanicolaou-stained. In the subjective morphological method, 200 spermatozoa per sample were assessed by a trained technician (Kruger's strict criteria) and the percentage of normal forms was recorded. The CASA morphometric analysis was performed with the SCA system (Microptic, Barcelona, Spain) according to the manufacturer recommendations. The slides were observed at $\times 1000$ magnification (Nikon Eclipse 50i, Egg, Switzerland). Size (length, width, area, perimeter) and shape (ellipticity, rugosity, elongation, regularity) of the sperm head, acrosome volume, mid-piece size and its insertion were recorded. The normality of the spermatozoon was inferred using a computer algorithm based on the Kruger's strict criteria. Statistical analysis was performed using STATA software (StataCorp, Texas, USA). Comparison between the two methods and the repeatability of each one was performed using linear regression analysis. The mean and standard deviation of each S-30

morphometric parameter were also determined.

Results: All samples ($n = 739$) were assessed with the CASA method. 308 smears were also analysed subjectively. Some slides were analysed twice in a blind fashion (30 by CASA and 19 by technician). The percentages of normal forms determined by the technician and the CASA were poorly correlated (slope \pm SD/y-intercept \pm SD/R²: $0.18 \pm 0.04/6.00 \pm 0.36/0.06$). The repeatability of the assessment was much better with the CASA system ($0.68 \pm 0.06/1.76 \pm 0.84/0.83$) than with the subjective determination ($0.35 \pm 0.11/4.70 \pm 1.00/0.38$). The measured morphometric parameters were close to the lower threshold of the WHO (range in parenthesis): length 4.2 ± 0.4 μm (4.0–5.5); width 2.7 ± 0.3 μm (2.5–3.5); ellipticity 1.6 ± 0.2 (1.5–1.75); acrosome volume $38 \pm 9\%$ (40–70).

Conclusion: Evaluation of sperm morphology using the CASA system can be performed by any qualified laboratory technician and does not require long specialized training. Furthermore, the CASA approach presents a better repeatability than the subjective method. In conclusion, CASA systems help reduce inter-laboratory variations and should allow a better understanding of sperm morphology effects on human infertility.

Production of monoclonal antibody to human spermatozoa with potent agglutination properties

Tabar MH¹, Ghaffari MA², Bijannejad D¹, Ghaforian M³, Samarbafzadeh AR⁴
¹Department of Anatomy; ²Department of Biochemistry; ³Department of Immunology; ⁴Department of Virology, School of Medicine, Jondishapour University of Medical Science

Objective: Antigenic features of spermatozoa are important for normal function, while there is some evidence that they are involved in naturally occurring anti-sperm antibodies (ASA) in men and women. There is some controversy regarding their role and relationship with infertility and sperm pathogenesis. There is no doubt about the sperm antigenic indexes; those that have potency to induce ASA with agglutination properties of spermatozoa are more important, because they negatively influence the fertilizing capacity of spermatozoa by impairment sperm-egg interaction and can inhibit the sperm motility and their penetration to egg. Production of monoclonal antibody against one of important function of spermatozoa (i.e. forward progression motility) is the aim of this study.

Materials/Methods: Normal human spermatozoa were used according to WHO criteria at $2 \times 10^7/\text{ml}$ and injected four times at 2-week intervals to immunize the mice. The spleen was removed aseptically from immunized mice and the splenocytes from one mouse were fused to NSO myeloma cells with 50% PEG solution methods. Standard sperm agglutination assay was performed to check that the positive hybridoma cells were adequate for cloning to produce monoclonal antibodies with positive sperm agglutination properties. The hybridoma cells were expanded from 96 wells to 175 cm^2 flasks and then the immunoglobulin (IgG) was purified from their supernatant by the saturated ammonium sulphate method.

Results: The fusion of immunized splenocytes and myeloma cells was carried out four times in almost 692 wells. There were 10 wells with positive sperm agglutination properties in fusion step and only one well (G2) in cloning step in which hybridoma cells were alive after three times serial dilution and could produce permanently antibodies with positive agglutination property. The morphology of agglutination test showed that all the spermatozoa treated with anti-sperm antibody attached to

each other head to head.

Conclusion: Production of monoclonal antibody against sperm motility can introduce diagnostic kits to distinguish pathological ASA from non-pathological cases and lead to better prognosis of ASA in patients suffering from infertility. This method can also screen the sperm surface epitopes and introduce a high potential antigenic inducer for pregnancy prevention as a immun contraceptive agent by means of vaccination against such a surface sperm epitopic indexes.

A role for mitochondrial respiration in human sperm hyperactivation as determined by a polarographic assay

Focarelli R², Stendardi A³, Piomboni P³, Coppola L⁴, Ferramosca A¹, Zara¹

¹Department of Biological and Environmental Sciences and Technologies, University of Salento, Lecce; ²Department of Evolutionary Biology; ³Department of Surgery, Biology Section, University of Siena, Siena Hospital, Siena; ⁴Technomed Medical Centre, Nardò, Lecce, Italy

Objective: Some studies have considered the possibility that mitochondrial dysfunction could be implicated as a factor in infertility; therefore a better clarification of the role of mitochondria in the function and in the overall metabolism of spermatozoa is desirable. A useful indicator of mitochondrial functionality is the oxygen consumption, since these organelles continuously oxidize different substrates reducing, at the same time, oxygen to water. In this study we report a simple method for analysing oxygen consumption and therefore mitochondrial functionality in human ejaculates.

Materials/Methods: We exclusively used human ejaculates in which semen analysis confirmed that all samples were normozoospermic according to the parameters of the World Health Organization having a progressive motility of 58 + 7.2% and a sperm concentration of $107 \pm 43 \times 10^6$ cells/ml, with fewer than one somatic cell per 10 optical fields ($\times 400$). Sperm cells were subjected to swim-up treatment to improve sperm quality. In order to test a possible correlation between sperm motility and/or the overall quality of human sperm, on the one hand, and the mitochondrial functionality, on the other hand, we measured the respiration capacity of human sperm mitochondria before and after swim-up treatment. Sperm cells were processed for hypotonic swelling and oxygen uptake by spermatozoa was measured at 36°C by using a Clark-type oxygen probe (Hansatech oxygraph, King's Lynn, UK), in the presence of 10 mmol/l malate and 10 mmol/l pyruvate and 0.76 μ mol/l ADP.

Results: The addition of pyruvate and L-malate to demembrated sperm cells before and after swim-up treatment promoted a significant oxygen uptake by mitochondria, which was further stimulated by ADP. The RCR (respiratory control ratio) values were calculated by dividing V3 (rate of oxygen uptake measured in the presence of substrates + ADP, i.e. state 3 of respiration) by V4 (rate of oxygen uptake measured with substrates alone, i.e. state 4 of respiration) and suggested good coupling between respiration and phosphorylation processes. However, in sperm samples selected by swim up, we found a significant increase of about 10-fold in the V3 and V4 values.

Conclusion: These results suggest that sperm motility strongly depends on mitochondrial respiratory function. Furthermore, high values of V3 and V4 obtained with sperm samples selected by swim up suggest that our experimental system responds also with a limited amount (less than 5×10^6)

of sperm cells. The results obtained open up new possibilities in biochemical and clinical studies. They will provide more insight into the basic biology of the spermatozoon with the aim of obtaining more information on the role of sperm mitochondria in motility and on the overall quality of the gametes.

Ovarian tissue grafting

Comparison study between intact and non-intact intramuscular autologous mouse ovaries

Siadat SF¹, Eimani H², Yazdi PE², Parivar K¹, Rezaazadeh M², Shahverdi A²

¹Department of Biology, Faculty of science, Science and Research branch, Islamic Azad University; ²Department of Embryology, Royan Institute, Tehran, Iran

Objective: Anticancer treatments often lead to ovarian failure and infertility. One of the solutions that have been adopted is cryopreservation and subsequent transplantation of the ovaries. One of the debates on this issue is primitive ischemia in the grafted ovary, which damages the oocyte pool.

Materials/Methods: In order to improve blood supply and follicle preservation, we made two incisions in ovaries before intramuscular auto-grafting and compared this group (non-intact ovaries) with intact ovaries that were also intramuscularly autografted. Follicle numbers and apoptosis were examined at 1, 2 and 3 weeks post-grafting in intact and non-intact groups. The results were compared with control ovaries that were not excised and grafted.

Results: We found that although follicle survival in both grafted groups was lower than controls ($P \leq 0.05$), survival of follicles in the grafted ovaries ($n = 19$) was improved by incising the ovary prior to grafting, compared with follicle survival in intact grafted ovaries.

Conclusion: However, estimated number of follicles decreased in grafted ovaries compared with non-grafted ovaries. Taken together, this procedure seems a promising method to preserve ovarian function but further studies are required to improve ovarian implant maintenance.

Read by title

Comparison of sperm concentration and sperm motility after 6 month intervals in the same patients

Aktas RG¹, Arat N¹, Ankarali H², Sofuoglu K¹, Cetinkaya T¹, Devranoglu B¹

¹ART Centre, Zeynep Kamil Women and Children Diseases Hospital, Istanbul; ²Department of Biostatistics, Faculty of Medicine, Karaelmas University, Zonguldak, Turkey

Role of recombinant human erythropoietin in the treatment of iron deficiency anaemia of pregnancy

Dimitrakopoulos S, Koliantzaki S, Sidiropoulou A, Goula K, Sorras K, Saltamavros A, Sidiropoulos N
General Hospital of Pyrgos, Obstetrics-Gynecology Department, Greece

ICSI on vitrified oocytes: our first childbirth

Palini S, De Stefani S, Polli V, Rocchi P, Tiezzi A, Bulletti C
Cervesi Hospital Cattolica, Rimini, Italy

Evolution in the policy of number of transferred embryos and multiple deliveries in IVF/ICSI programme. Register of the Spanish Fertility Society 2004

Cabello Y, Castilla JA, Hernández J, Marqueta J, Coroleu B
Spanish Fertility Society, Spain

Selection of embryos to transfer using combined embryo quality and zygote pronuclear morphology as a score in a new IVF centre

Cabello Y, García-Enguádanos A, Roca P, Santaolaya A
Fiv recoletos

Screens of optical depiction and work in pregnancy

Dimitrakopoulos S¹, Bonas A¹, Goula K¹, Saltamavros A¹, Sidiropoulou A¹, Sorras K¹, Koliantzaki S¹, Sidiropoulos N¹, Koutos C²

¹General Hospital of Pyrgos, Obstetrics-Gynecology Department; ²Occupational Health Centre West of Greece

Metre of precaution of frail teams of workers from the emission electromagnetic radiation in the labour spaces

Dimitrakopoulos S¹, Koliantzaki S¹, Sidiropoulou A¹, Bonas A¹, Mpoumpoulas V¹, Goula K², Saltamavros A², Sidiropoulos N¹, Stergiopoulos C³, Koutos C³

¹Obstetrics-Gynecology Department, General Hospital of Pyrgos; ²Department of Medicine, Saint Andrew Hospital, Patras; ³Occupational Health Centre West of Greece

Changes in endometrial indexes of ovariectomized mice in response to exogenous ovarian hormones

Peyghambari F¹, Salehnia M¹, Moghadam MF¹, Valujerdi MR¹, Hajizadeh E¹

¹Department of Anatomy; ²Department of Biotechnology; ³Department of Biostatistic, Tarbiat Modares University, Iran

Early cleavage stage versus blastocyst stage embryo transfer in IVF-embryo transfer cycles

Karaarslan F, Aslan D, Keles R, Demir N
IRENBE Reproductive Health Centre

Fresh embryo transfer cancellation as a strategy to reduce the risk of ovarian hyperstimulation syndrome

Prados F, Pérez-Bermejo G, Pinto S, Díaz A, Collado O, Sánchez de Rivera M, Bruna I
Hospital de Madrid Montepríncipe

Role of recombinant human erythropoietin in the treatment of iron deficiency anaemia of pregnancy

Dimitrakopoulos S, Koliantzaki S, Sidiropoulou A, Sorras K, Saltamavros A, Goula K, Tseliou P, Sidiropoulos N
Obstetrics-Gynecology Department, General Hospital of Pyrgos, Greece

Gestation-increased cervical transparency and biochemical indicators

Dimitrakopoulos S, Koliantzaki S, Sidiropoulou A, Sorras K, Saltamavros A, Tseliou P, Sidiropoulos N
Obstetrics-Gynecology Department, General Hospital of Pyrgos, Greece

Endometriosis is associated with lower number of transferred embryos and decreased fertilization rates

Ozsait B, Bulgurcuoglu S, Erkan Y, Attar E, Serdaroglu H
IVF Unit, Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Istanbul University, Istanbul, Turkey

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