Acta Scientiae Veterinariae, 2011. 39(Suppl 1): s47 - s55.

ISSN 1679-9216 (Online)

State of the Art of Assisted Human Reproduction

Eduardo Leme Alves da Motta[1,2], Marcilio Nichi[1] & Paulo Cesar Serafini[1,3]

ABSTRACT

***Background*:** Infertility is a disease observed in approximately 10% of the reproductive age population (20-44 years old), and is defined as the failure to conceive after twelve months of regular sexual intercourse, without contraception; in women older than 35 years old, this period is reduced to 6 months. The main causes of infertility are tubal, ovarian and uterine and sperm abnormalities, endometriosis, and those with undetermined causes. Over the past 30 years, several techniques were developed to overcome these factors including gamete cryopreservation, controlled ovarian stimulation, intra-uterine insemination, *in vitro* fertilization, intracytoplasmatic sperm injection).

***Review*:** Despite advances in assisted reproductive technologies (ART), treatment success is still strongly dependent on oocyte and sperm quality, and resulting embryo viability. The most promising advance on oocyte quality assessment is the evaluation of the ovarian reserve by the quantification of the anti-müllerian hormone (AMH). Since ovarian reserve is closely related to oocyte quality, AMH levels could be an indicator of both oocyte production capacity and the potential of these oocytes to generate a viable embryo. On the other hand, despite the development of techniques to overcome male factor infertility, attention has been paid on the semen evaluation, since routine sperm evaluation techniques are known to be ineffective, especially in those cases of unexplained infertility. Therefore, techniques were developed to assess acrosome and membrane integrity, mitochondrial potential, DNA integrity, and fertilizing capacity of sperm. However, further studies are necessary to evaluate sperm DNA integrity without damaging the cell, allowing the injection of a spermatozoon with an intact DNA when using ICSI. Regarding embryo quality, even with a good quality oocyte (as assessed by the current techniques) and an apparently normal sperm, there are still chances of generating an embryo with genetic abnormalities. In such cases, and in cases of recurrent failures, women over 35 years of age, and couples with a pre-existing genetic risk, the preimplantation genetic diagnosis (PGD) appears to be an important tool to improve the odds of pregnancy and avoid abortions or the conception of fetuses with genetic abnormalities. The technique of PGD, usually performed with PCR or FISH, has gained a powerful tool with the development of the Comparative Genomic Hybridization (CGH). However, recent studies aiming to identify markers of oocyte and sperm quality and embryo viability are in course using mass spectroscopy. With this sensitive technique applied to body fluids (i.e., blood, follicular fluid, seminal plasma), granulosa cells, sperm, and culture media, researches are being conducted to non-invasively identify biomarkers that will help understand reproductive mechanisms and to efficiently predict the outcome of ARTs.

***Conclusion*:** Significant advances in ART have been observed in the last few years, yet, failures still occur with high frequency. This review will focus on techniques to assess oocyte quality, sperm function and embryo viability, aiming to provide tools for a precise prognosis when treating infertile couples.

Keywords: Assisted reproductive technologies, anti-müllerian hormone, sperm function tests, preimplantation genetic diagnosis, comparative genomic hybridization, omics.

[1]Huntington Medicina Reprodutiva, São Paulo, SP, Brazil. [2]Disciplina de Ginecologia, Universidade Federal de São Paulo, São Paulo, SP, Brazil. [3]Departamento de Ginecologia, Faculdade de Medicina da Universidade de São Paulo (USP), São Paulo, SP, Brazil. CORRESPONDENCE: E.L.A. Motta [emotta@huntington.com.br]. Huntington Medicina Reprodutiva, Av. República do Líbano n. 529, CEP 04501000 São Paulo, SP, Brazil.

I. INTRODUCTION

Infertility was defined as a disease for the first time in 2008 by the Practice Committee of the American Society for Reproductive Medicine [48]. Soon after, the World Health Organization (WHO) also defined infertility as a disease, publishing simultaneously in Human Reproduction [78] and Fertility and Sterility [79]. The modern societal trend of women trying to conceive after 35 years of age, combined with the dramatic and irreversible decrease in oocyte potential with increased age, has lead to more couples facing infertility issues. Therefore, in the near future, medical insurance companies will likely be obliged to cover the costs of assisted reproduction techniques (ART).

Although considerable advances have been achieved in recent years, many new technologies to improve the fertility rate will likely be introduced in the near future. A successful human pregnancy depends on several factors, such as ovarian function, sperm quality, embryo development, and a receptive endometrium; these factors, alone or combined, strongly influence ART results [5]. The purpose of this review is to briefly describe some of the most recent advances in the infertility field.

II. OOCYTE

The oocyte is well known to be the key player in reproduction outcome, however, techniques to evaluate fertility of the female gamete are still poor. Assessment of the ovarian reserve prior to ART is essential to predict outcome, select the best stimulation protocol, and retrieve the most viable oocytes in order to increase the success rate of the treatments employed. Furthermore, studies indicate that poor ovarian reserve is also accompanied by a decrease in oocyte quality [32]. Antral follicle count by ultrasound [17], early (basal) measures of follicle stimulating hormone (FSH) [1]), estradiol (E_2, [77]), and inhibin B [24] levels, as well as the clomiphene citrate challenge test [49] remain the standard evaluations. Also, increased female age correlates with diminished ovarian function [16], and may be used as an indicator of ovarian reserve. However, when performed separately, the above mentioned tests are not efficient to reflect ovarian status [23]. A promising tool to evaluate ovarian reserve is assessment of anti-Müllerian hormone (AMH) levels. AMH is produced in Sertoli cells and was previously believed to function only in the differentiation of males by inducing regression of the Müllerian ducts, the primordial anlage of the female reproductive tract [44]. AMH is a dimeric glycoprotein of the transforming growth factor family (TGF) known to regulate cell growth and differentiation [50]. This hormone appears to play an inhibitory role in the early recruitment, selection, and growth of primordial follicles and in cyclic FSH-induced antral and pre-antral follicular growth [75]. Granulosa cells of pre-antral and antral follicles are known to produce AMH. Collectively, these data indicate that this hormone may be a potential marker to predict the state of the ovarian primordial follicle reserve. In fact, several studies have shown the efficacy of AMH level testing, alone or combined with ultrasound antral follicle count, on predicting ovarian responsiveness to stimulation protocols when compared to traditional procedures [26,58,71].

III. SPERMATOZOA

Semen analysis (SA) is used worldwide to assess male fertility and is mainly focused on sperm concentration, progressive motility, and morphology; WHO recently published a new reference value for fertile men based on these variables (Table 2) [18]. These values are based on the fifth centile found in a population of fertile men (men with a formerly pregnant partner with a time-to-pregnancy ≤ 12 months). However, despite studies indicating the efficiency of sperm count and morphology assessment as predictors of pregnancy likelihood [14], SA has some well known limitations since it represents the ability to fertilize an egg, not the ability to produce a viable embryo. In fact, the current WHO standard for normozoospermia does not accurately predict subfertile men [4], which was found to contribute to almost 40% of couples presenting with unexplained infertility [70]. Furthermore, with the advent of intracytoplasmic sperm injection (ICSI), the assessment of sperm motility, concentration, and morphology may be obsolete. In the last decade, new

Table 1. Ovarian reserve markers.

Marker	Advantages	Limitations
Age	Correlates with several reproductive outcomes	High variability between women with the same age
Antral follicle count	Gold standard, performed by ultrasound (non-invasive)	Subjective, intra-observer variations, response dependent on LH levels
FSH on day 3	Routinely used, may reflect the ability of the particular month and not the overall reproductive ability	Variation between immunoassays, inter- and intra-cycle variations, feedback influence
Inhibin B	Correlates with response to gonadotropin	High false-positive rate, not widely available, feedback influence
Clomiphene challenge test	Good predictor of ovarian response, not IVF outcome	Time consuming, depends on FSH immunoassay
AMH	Low variation between cycles and individuals, not affected by GnRH agonists	Not widely available

Table 2. Reference values for semen characteristics – WHO 2010 [18].

Semen Variable	Reference values
Volume	> 1.5 mL
Sperm concentration	> 15 million spermatozoa/mL
Total sperm count	At least 39 million spermatozoa/ejaculate
Progressive motility	> 32% of progressive spermatozoa
Strict Morphology	> 4.0% of normal spermatozoa

techniques for semen analysis have been developed based on routine methods with promising results, such as computer-assisted sperm analysis (CASA; [47]) and SuperICSI (ICSI using sperm specifically selected by high magnification microscopy; [35]). However, new techniques are necessary to not only determine sperm fertilization capacity, but also functional abilities, and are essential for diagnostic and prognostic purposes in helping to determine the most efficient treatment and to select the ideal gamete to be used.

3.1 Sperm functionality tests

Studies indicate that sperm functional competence rather than the number of motile and morphologically normal cells is determinant to predict sperm fertility (22). Therefore, techniques to evaluate sperm membrane integrity, acrosome status, mitochondrial activity, DNA integrity and sperm fertilizing capacity have been developed (Table 3).

3.1.1 Membrane integrity (vitality)

The integrity of the sperm plasma membrane is essential to protect the DNA from injury during fertilization. Also, reported data indicates that a number of motile cells have a disrupted membrane; the identification of such cells would be extremely beneficial in increasing the efficiency of ART.

Several techniques to evaluate sperm membrane integrity are available. Most dye-exclusion techniques can accurately identify sperm with a damaged membrane in the ejaculate, including techniques developed more than 60 years ago (eosin and nigrosin [11]) to the more recently developed fluorescent probes (propidium iodide [25] and Hoechst 33258 [56]). This information is critical when analyzing the semen sample for IVF. However, most of the established techniques kill the spermatozoa, preventing its use for ICSI. Therefore, the ideal technique to test sperm membrane integrity is the hypo-osmotic swelling test, which results in tail swelling of spermatozoa with damaged membranes while leaving normal spermatozoa intact.

3.1.2 Acrosome status

The acrosome, an organelle formed from the

Table 3. Sperm function tests.

Sperm function	Commercially available tests
Plasma Membrane	Hyposmotic swelling test, eosin-nigrosin, Propidium iodide, Hoechst 33258
Acrosome	Arachis hypogaea agglutinin, Pisum sativum agglutinin, Trypan Blue/Bengal rose, Hoechst 33258
Mitochondria	JC1, Rhodamine 123, Mito tracker, 3,3'-diaminobenzidine
DNA fragmentation	Comet assay, TUNEL, Sperm Chromatin Structure Assay, CMA3
Penetration assays	Zona-free hamster oocyte, binding to egg yolk membrane, hyaluronic acid assay

Golgi complex that develops over the anterior head of sperm, is required for binding of sperm to the oocyte and contains proteolytic enzymes that digest through the zona pellucida (acrosome reaction) [73]. The effect of the acrosome reaction on the fertilization process is well established. Therefore, assessment of acrosome integrity is important in determining subfertility, particularly after failure of multiple IVF cycles. Furthermore, since acrosome and membrane integrity are closely related, the assessment of acrosome integrity is usually accompanied by a vitality test.

Acrosome integrity can be assessed by fluorescent lectins that bind to the acrosomal membrane (*Arachis hypogaea agglutinin*) or to the acrosomal contents (*Pisum sativum agglutinin*) [45]. Trypan Blue [68] and Hoechst 33258 [56] staining have also been successfully used to accurately detect acrosome integrity. A technique that is frequently used to evaluate the potential of the acrosome to react is to induce the acrosome reaction with ionophore A23187 [19], progesterone [15], human zona pellucida [57], or zona-free hamster oocytes [76].

3.1.3 Mitochondrial potential

A number of enzymes responsible for anaerobic glycolysis to generate ATP for sperm motility were identified in the sperm tail [54]. However, mitochondrial ATP produced by oxidative phosphorylation has been shown to play an essential role in flagellar movement [6]. Additionally, sperm mitochondria are important in the regulation of oxidative stress and apoptosis [8,46]. Therefore, the functional properties of the mitochondria likely affect the fertility potential of the sperm.

The assessment of mitochondrial status is usually performed using fluorescent probes, such as Rhodamine 123, JC-1, and MitoTracker red, green, or orange, each having their own advantages and limitations [33]. However, all tests require a fluorescent microscope or a flow cytometer (sometimes with more than one color detector) for the assay, which improves the efficiency of the technique, but limits the use for most ART centers. An alternative method is the use of 3,3'-diaminobenzidine (DAB); the oxidation of DAB by cytochrome c oxidase forms a brown complex and may reflect sperm mitochondria activity [39]. The grade of staining in the sperm midpiece can be visualized by contrast microscopy and correlated with the level of mitochondrial activity [12].

3.1.4 DNA integrity

The spermatozoa should carry an intact male genome to properly activate an oocyte during the fertilization process [65]. Studies indicate that even with fragmented DNA the spermatozoa may correctly fertilize the egg [5,52], if such abnormalities are below a critical threshold [2]. However, depending on the extent of the DNA fragmentation, embryo potential may be affected [3], leading to impaired development, low implantation rates, miscarriages, and likely contribute to abnormalities in the offspring [9, 53].

Several tests have been developed to assess sperm DNA fragmentation, and the results are reported, generally, as "DNA damage". However, the different tests can distinguish distinct properties of the DNA, determining several aspects of the DNA abnormality. While the Terminal Transferase dUTP Nick End Labeling assay (TUNEL) assesses the 'real' DNA damage, the Sperm Chromatin Structure Assay (SCSA) evaluates the 'potential' DNA damage in terms of susceptibility to DNA denaturation [36]. On the other hand, the Comet assay, when performed at neutral pH, is able to detect double-strand breaks [64], and when performed at acidic or alkaline pH, detects single-stranded breaks [61]. This distinction is important since single-stranded breaks are easier to repair than double-stranded breaks. Therefore, care should be taken when counseling patients and making recommendations based on the results of a sperm DNA test. According to Sakkas and Alverez [61], only a

combination of tests can properly evaluate different aspects of the DNA damage (i.e., single versus double--stranded breaks, real versus induced fragmentation).

3.1.5 Penetration assays

The sperm penetration assays is probably the most effective technique to evaluate sperm fertilizing capacity, since it simultaneously evaluates the sperms' ability to capacitate, undergo the acrosome reaction, and fuse with the vitelinic membrane of an oocyte.

Studies performed with animal and artificial models, such as the zona-free hamster oocyte [38], egg yolk membrane [10], and the hyaluronic acid binding assay [41], suggest that these tests are accurate in determining the ability of sperm to bind to the oocyte. Furthermore, the number of sperm with chromosomal disomy is decreased in hyaluronic acid treated sperm, which allows for selection of healthy, mature sperm for ICSI.

IV. EMBRYO

The embryonic potential to invade and attach at the endometrial cavity still remains the critical event to achieve a viable pregnancy. Previous studies indicate that genetic abnormalities are involved in 50-80% of first-trimester miscarriages [31,62], 20-30% of neonatal deaths [40], [20], and 30-50% of post-natal deaths [37]. Furthermore, genetic abnormalities were observed in 50% of mentally retarded patients [42], 10% of cancer patients [28], and are linked with many other diseases. Until recently, the diagnosis of genetic abnormalities was based exclusively on results from nonspecific ultrasonographic findings [67], maternal blood tests [22,63], amniocentesis [63], and chorionic villus sampling [29], which are performed after implantation occurs. Since termination of the pregnancy is complicated by ethical and legal concerns [43], the antenatal diagnosis of a genetically abnormal fetus would be a better choice to select the most viable embryo. The pre-implantation genetic diagnosis (PGD) procedure was developed more than 20 years ago, which allowed for the genetic testing of embryos before implantation [34], selecting only healthy embryos for transfer.

PGD analyzes the polar body in the oocyte, the cleavage stage embryo, or the blastocyst stage embryo by genetic analysis, which can be performed by polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH), and, more recently, by comparative genomic hybridization (CGH). PCR analysis is used to test for monogenic diseases, such as cystic fibrosis [51], Huntington disease [66], fragile X syndrome [7], and Duchenne muscular dystrophy [51]. FISH analysis is used to detect structural (deletion, duplication, and translocation) and numerical (trysomy and monosomy) chromosomal abnormalities which result in diseases, such as Down syndrome [80], Turner's syndrome [55], and Klinefelter's syndrome [59]. However, despite continuous advances, these techniques can only identify a limited number of genetic abnormalities. With the development of CGH, it is now possible to perform a screening of the whole genome to identify an unbalanced number of chromosomal copies [74].

V. PERSPECTIVES

Over the past couple decades, several studies have been performed aiming to identify and quantify "substances" that could elucidate biological mechanisms and explain differences between individuals, races, and species. The term "biomarker" was then created to refer to such substances that indicate normal biological processes, pathogenic processes, or responses to the environment or therapeutic intervention. Since the sequencing of the human genome [72], several studies were performed to better characterize the function and interaction between key biomolecules, such as proteins (proteome), DNA transcripts (transcriptome), lipids (lipidome), and metabolites (metabolome), the so-called "Omics" approach. However, care should be taken when correlating these results with the biological process since the state of the genome may not reflect the transcripts generated [27], changes in mRNA do not reflect absolute or relative changes in protein levels [69], and the proteins produced may not reflect the biological process and phonotypical characteristics [60]. A revolution has been observed in the fields of proteome, lipidome, and metabolome research with the development of more sensitive and selective techniques, including mass spectrometry, which, in contrast to previously used techniques, allows the screening of thousands of proteins, peptides, and small molecules (metabolites and lipids) [30]. In reproduction, an Omics approach applied to follicular fluid, granulosa cells, embryo culture media, seminal plasma, and other biological fluids may lead to the identification of biomarkers that elucidate several still unknown mechanisms related to the oocyte, spermatozoa, and/or embryo, and is a non-invasive test that may identify couples with higher odds of achieving pregnancy.

REFERENCES

1 Abdalla H. & Thum M.Y. 2004. An elevated basal FSH reflects a quantitative rather than qualitative decline of the ovarian reserve. *Human Reproduction*. 19(4): 893-898.

2 Ahmadi A. & Ng S.C. 1999. Developmental capacity of damaged spermatozoa. *Human Reproduction*. 14(9): 2279-2285.

3 Aitken R.J. & Baker M.A. 2006. Oxidative stress, sperm survival and fertility control. *Molecular and Cellular Endocrinology*. 250(1-2): 66-69.

4 Aitken R.J. 2006. Sperm function tests and fertility. *International Journal of Andrology*. 29(1): 69-75; discussion 105-108.

5 Aitken R.J., Gordon E., Harkiss D., Twigg J.P., Milne P., Jennings Z. et al. & D. Irvine S. 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biology of Reproduction*. 59(5): 1037-1046.

6 Amann R.P. 1989. Can the fertility potential of a seminal sample be predicted accurately? *Journal of Andrology*. 10(2): 89-98.

7 Apessos A., Abou-Sleiman P.M., Harper J.C. & Delhanty J.D. 2001. Preimplantation genetic diagnosis of the fragile X syndrome by use of linked polymorphic markers. *Prenatal Diagnosis*. 21(6): 504-511.

8 Aziz N., Said T., Paasch U. & Agarwal A. 2007. The relationship between human sperm apoptosis, morphology and the sperm deformity index. *Human Reproduction*. 22(5): 1413-1419.

9 Baker M.A. & Aitken R.J. 2005. Reactive oxygen species in spermatozoa: methods for monitoring and significance for the origins of genetic disease and infertility. *Reproductive Biology and Endocrinology*. 3: 67.

10 Barbato G.F., Cramer P.G. & Hammerstedt R.H. 1998. A practical *in vitro* sperm-egg binding assay that detects subfertile males. *Biology of Reproduction.* 58(3): 686-699.

11 Blom E. 1950. A one-minute live-dead stain by means of eosin-nigrosin. *Fertility and Sterility*. 1: 2.

12 Blumer C.G., Fariello R.M., Restelli A.E., Spaine D.M., Bertolla R.P. & Cedenho A.P. 2008. Sperm nuclear DNA fragmentation and mitochondrial activity in men with varicocele. *Fertility and Sterility*. 90(5): 1716-1722.

13 Boivin J., Bunting L., Collins J.A. & Nygren K.G. 2007. International estimates of infertility prevalence and treatment--seeking: potential need and demand for infertility medical care. *Human Reproduction*. 22(6): 1506-1512.

14 Bonde J.P.E., Ernst E., Jensen T.K., Hjollund N.H.I., Kolstad H., Scheike T., Giwercman A., Skakkebæk N.E., Henriksen T.B. & Olsen J. 1998. Relation between semen quality and fertility: a population-based study of 430 first--pregnancy planners. *The Lancet*. 352(9135): 1172-1177.

15 Bronson R.A., Peresleni T. & Golightly M. 1999. Progesterone promotes the acrosome reaction in capacitated human spermatozoa as judged by flow cytometry and CD46 staining. *Molecular Human Reproduction*. 5(6): 507-512.

16 Chen S.L., Xia R., Chen X., Luo Y.Q., Wang L.L., Wu Y.Q., Shi X.Y. & Zheng H.Y. 2011. Prediction of ovarian reserve, poor response and pregnancy outcome based on basal antral follicle count and age in patients undergoing *in vitro* fertilization-embryo transfer. *Nan Fang Yi Ke Da Xue Xue Bao*. 31(4): 572-577.

17 Chen W.H., Lu Y.W., Lai F., Chien Y.H. & Hwu W.L. 2011. Integrating Human Genome Database into Electronic Health Record with Sequence Alignment and Compression Mechanism. *Journal of Medical Systems*. [in press].

18 Cooper T.G., Noonan E., von Eckardstein S., Auger J., Baker H.W., Behre H.M., Haugen T.B., Kruger T., Wang C., Mbizvo M.T. & Vogelsong K.M. 2010. World Health Organization reference values for human semen characteristics. *Human Reproduction Update.* 16(3): 231-245.

19 Cummins J.M., Pember S.M., Jequier A.M., Yovich J.L. & Hartmann P.E. 1991. A test of the human sperm acrosome reaction following ionophore challenge. Relationship to fertility and other seminal parameters. *Journal of Andrology*. 12(2): 98-103.

20 Cunniff C., Carmack J.L., Kirby R.S. & Fiser D.H. 1995. Contribution of heritable disorders to mortality in the pediatric intensive care unit. *Pediatrics*. 95(5): 678-681.

21 DeCherney A.H. 1986. *In vitro* fertilization and embryo transfer: a brief overview. *Yale Journal of Biology and Medicine*. 59(4): 409-414.

22 Driscoll D.A. & Gross S.J. Screening for fetal aneuploidy and neural tube defects. *Genetics in Medicine.* 11(11): 818-821.

23 Eldar G.T., Ben C.A., M.Spitz I., Rabinowitz R., Markowitz E., Mimoni T., Gal M., Zylber H.E. & Ehud J.M. Dynamic assays of inhibin B, anti-Mullerian hormone and estradiol following FSH stimulation and ovarian ultrasonography as predictors of IVF outcome. *Human Reproduction*. 20(11): 3178-3183.

24 Erdem M., Erdem A., Gursoy R. & Biberoglu K. 2004. Comparison of basal and clomiphene citrate induced FSH and inhibin B, ovarian volume and antral follicle counts as ovarian reserve tests and predictors of poor ovarian response in IVF. *Journal of Assisted Reproduction and Genetics*. 21(2): 37-45.

25 Falzone N., Huyser C. & Franken D.R. 2010. Comparison between propidium iodide and 7-amino-actinomycin-D for viability assessment during flow cytometric analyses of the human sperm acrosome. *Andrologia.* 42(1): 20-26.

26 Ficicioglu C., Kutlu T., Baglam E. & Bakacak Z. 2006. Early follicular antimullerian hormone as an indicator of ovarian reserve. *Fertility and Sterility*. 85(3): 592-596.

27 Forrest A.R. & Carninci P. 2009. Whole genome transcriptome analysis. *RNA Biology*. 6(2): 107-112.

28 Frank S.A. 2004. Genetic predisposition to cancer - insights from population genetics. *Nature Reviews Genetics*. 5(10): 764-772.

29 Goumy C., Bonnet-Dupeyron M.N., Cherasse Y., Laurichesse H., Jaffray J.Y., Lacroute G., Geneix A., Lemery D. & Vago P. 2004. Chorionic villus sampling (CVS) and fluorescence *in situ* hybridization (FISH) for a rapid first--trimester prenatal diagnosis. *Prenatal Diagnosis*. 24(4): 249-256.

30 Griffiths W.J. & Wang Y. 2009. Mass spectrometry: from proteomics to metabolomics and lipidomics. *Chemical Society Reviews*. 38(7): 1882-1896.

31 Guerneri S., Bettio D., Simoni G., Brambati B., Lanzani A. & Fraccaro M. 1987. Prevalence and distribution of chromosome abnormalities in a sample of first trimester internal abortions. *Human Reproduction*. 2(8): 735-739.

32 Haadsma M.L., Groen H., Mooij T.M., Burger C.W., Broekmans F.J., Lambalk C.B., van Leeuwen F.E., Hoek A. 2010. Miscarriage risk for IVF pregnancies in poor responders to ovarian hyperstimulation. *Reproductive BioMedicine Online*. 20(2): 191-200.

33 Hallap T., Nagy S., Jaakma U., Johannisson A. & Rodriguez-Martinez H. 2005. Mitochondrial activity of frozen--thawed spermatozoa assessed by MitoTracker Deep Red 633. *Theriogenology*. 63(8): 2311-2322.

34 Handyside A.H., Kontogianni E.H., Hardy K. & Winston R.M. 1990. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*. 344(6268): 768-770.

35 Hazout A., Dumont-Hassan M., Junca A.M., Cohen Bacrie P. & Tesarik J. 2006. High-magnification ICSI overcomes paternal effect resistant to conventional ICSI. *Reproductive BioMedicine Online*. 12(1): 19-25.

36 Henkel R., Hoogendijk C.F., Bouic P.J. & Kruger T.F. 2010. TUNEL assay and SCSA determine different aspects of sperm DNA damage. *Andrologia*. 42(5): 305-313.

37 Hoekelman R.A. & Pless I.B. 1988. Decline in mortality among young Americans during the 20th century: prospects for reaching national mortality reduction goals for 1990. *Pediatrics*. 82(4): 582-595.

38 Hough S.R., Kaproth M.T. & Foote R.H. 2002. Induction of the acrosome reaction and zona-free hamster oocyte penetration by a bull with complete teratospermia versus a half brother with normal sperm. *Journal of Andrology*. 23(1): 98-106.

39 Hrudka F. 1987. Cytochemical and ultracytochemical demonstration of cytochrome c oxidase in spermatozoa and dynamics of its changes accompanying ageing or induced by stress. *International Journal of Andrology*. 10(6): 809-828.

40 Hudome S.M., Kirby R.S., Senner J.W. & Cunniff C. 1994. Contribution of genetic disorders to neonatal mortality in a regional intensive care setting. *American Journal of Perinatology.* 11(2): 100-103.

41 Huszar G., Ozkavukcu S., Jakab A., Celik-Ozenci C., Sati G.L. & Cayli S. 2006. Hyaluronic acid binding ability of human sperm reflects cellular maturity and fertilizing potential: selection of sperm for intracytoplasmic sperm injection. *Current Opinion in Obstetrics and Gynecology*. 18(3): 260-267.

42 Inlow J.K. & Restifo L.L. 2004. Molecular and comparative genetics of mental retardation. *Genetics*. 166(2): 835-881.

43 Jones K. & Chaloner C. 2007. Ethics of abortion: the arguments for and against. *Nurs Stand*. 21(37): 45-48.

44 Josso N., Cate R.L., Picard J.Y., Vigier B., di Clemente N., Wilson C., Imbeaud S., Pepinsky R.B., Guerrier D. & Boussin L. 1993. Anti-mullerian hormone: the Jost factor. *Recent Progress in Hormone Research*. 48: 1-59.

45 Kohn F.M., Mack S.R., Schill W.B. & Zaneveld L.J. 1997. Detection of human sperm acrosome reaction: comparison between methods using double staining, *Pisum sativum agglutinin*, concanavalin A and transmission electron microscopy. *Human Reproduction*. 12(4): 714-721.

46 Koppers A.J., De Iuliis G.N., Finnie J.M., McLaughlin E.A. & Aitken R.J. 2008. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *The Journal of Clinical Endocrinology & Metabolism*. 93(8): 3199-3207.

47 Krause W. 1995. Computer-assisted semen analysis systems: comparison with routine evaluation and prognostic value in male fertility and assisted reproduction. *Human Reproduction*. 10 (Suppl 1): 60-66.

48 Ku L. 2008. Terminology Tuesdays: Definitions of infertility and recurrent pregnancy loss. *Fertility and Sterility*. 90 (Suppl-5): S60.

49 Kwee J., Schats R., McDonnell J., Schoemaker J. & Lambalk C.B. 2006. The clomiphene citrate challenge test versus the exogenous follicle-stimulating hormone ovarian reserve test as a single test for identification of low responders and hyperresponders to *in vitro* fertilization. *Fertility and Sterility*. 85(6): 1714-1722.

50 Lee M.M., Donahoe P.K., Hasegawa T., Silverman B., Crist G.B., Best S., Hasegawa Y., Noto R.A., Schoenfeld D. & MacLaughlin D.T. 1996. Mullerian inhibiting substance in humans: normal levels from infancy to adulthood. *The Journal of Clinical Endocrinology & Metabolism*. (2): 571-576.

51 Liu J., Lissens W., Devroey P., Liebaers I. & Van Steirteghem A. 1996. Cystic fibrosis, Duchenne muscular dystrophy and preimplantation genetic diagnosis. *Human Reproduction Update*. 2(6): 531-539.

52 Lopes S., Sun J.G., Jurisicova A., Meriano J. & Casper R.F. 1998. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertility and Sterility*. 69(3): 528-532.

53 Marchetti F. & Wyrobek A.J. 2005. Mechanisms and consequences of paternally-transmitted chromosomal abnormalities. *Birth Defects Research Part C: Embryo Today*. 75(2): 112-129.

54 Narisawa S., Hecht N.B., Goldberg E., Boatright K.M., Reed J.C. & Millan J.L. 2002. Testis-specific cytochrome c-null mice produce functional sperm but undergo early testicular atrophy. *Molecular and Cellular Biology.* 22(15): 5554-5562.

55 Onalan G., Yilmaz Z., Durak T., Sahin F.I. & Zeyneloglu H.B. 2011. Successful pregnancy with preimplantation genetic diagnosis in a woman with mosaic Turner syndrome. *Fertility and Sterility*. 95(5): 1788 e1-3.

56 Ozaki T., Takahashi K., Kanasaki H. & Miyazaki K. 2002. Evaluation of acrosome reaction and viability of human sperm with two fluorescent dyes. *Archives of gynecology and obstetrics journal*. 266(2): 114-117.

57 Patrat C., Auer J., Fauque P., Leandri R.L., Jouannet P. & Serres C. 2006. Zona pellucida from fertilised human oocytes induces a voltage-dependent calcium influx and the acrosome reaction in spermatozoa, but cannot be penetrated by sperm. *BMC Developmental Biology.* 6: 59.

58 Peñarrubia J., Fábregues F., Manau D., Creus M., Casals G., Casamitjana R., Carmona F., Vanrell J.A. & Balasch J. 2005. Basal and stimulation day 5 anti-Mullerian hormone serum concentrations as predictors of ovarian response and pregnancy in assisted reproductive technology cycles stimulated with gonadotropin-releasing hormone agonist--gonadotropin treatment. *Human Reproduction*. 20(4): 915-922.

59 Reubinoff B.E., Abeliovich D., Werner M., Schenker J.G., Safran A. & Lewin A. 1998. A birth in non-mosaic Klinefelter's syndrome after testicular fine needle aspiration, intracytoplasmic sperm injection and preimplantation genetic diagnosis. *Human Reproduction*. 13(7): 1887-1892.

60 Saghatelian A. & Cravatt B.F. 2005. Global strategies to integrate the proteome and metabolome. *Current Opinion in Chemical Biology*. 9(1): 62-68.

61 Sakkas D. & Alvarez J.G. 2010. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertility and Sterility*. 93(4): 1027-1036.

62 Schaeffer A.J., Chung J., Heretis K., Wong A., Ledbetter D.H. & Lese Martin C. 2004. Comparative genomic hybridization-array analysis enhances the detection of aneuploidies and submicroscopic imbalances in spontaneous miscarriages. *American Journal of Human Genetics.* 74(6): 1168-1174.

63 Sekizawa A., Purwosunu Y., Matsuoka R., Koide K., Okazaki S., Farina A., Saito H. & Okai T. 2007. Recent advances in non-invasive prenatal DNA diagnosis through analysis of maternal blood. *Journal of Obstetrics and Gynaecology Research*. 33(6): 747-764.

64 Singh N.P., McCoy M.T., Tice R.R. & Schneider E.L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*. 175(1): 184-191.

65 Smith R, Kaune H, Parodi D, Madariaga M, Rios R, Morales I. & Castro A. 2006. Increased sperm DNA damage in patients with varicocele: relationship with seminal oxidative stress. *Human Reproduction*. 21(4): 986-993.

66 Stern H.J., Harton G.L., Sisson M.E., Jones S.L., Fallon L.A., Thorsell L.P., Getlinger M.E., Black S.H. & Schulman J.D. 2002. Non-disclosing preimplantation genetic diagnosis for Huntington disease. *Prenatal Diagnosis*. 22(6): 503-507.

67 Stewart T.L. 2004. Screening for aneuploidy: the genetic sonogram. *Obstetrics & Gynecology Clinics of North America.* 31(1): 21-33.

68 Talbot P. & Chacon R.S. 1981. A triple-stain technique for evaluating normal acrosome reactions of human sperm. *Journal of Experimental Zoology*. 215(2): 201-208.

69 Unwin R.D. & Whetton A.D. 2006. Systematic proteome and transcriptome analysis of stem cell populations. *Cell Cycle*. 5(15): 1587-1591.

70 Van der Steeg J.W., Steures P., Eijkemans M.J.C., Habbema J.D.F., Hompes P.G., Kremer JA, van der Leeuw--Harmsen L., Bossuyt P.M.M., Repping S., Silber S.J., Ben W.J. & van der Veen F. 2011. Role of semen analysis in subfertile couples. *Fertility and Sterility*. 95(3): 1013-1019.

71 Van Rooij I.A., Broekmans F.J., te Velde E.R., Fauser B.C., Bancsi L.F.J.M.M., Jong F.H. & Themmen A.P.N. 2002. Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. *Human Reproduction*. 17(12): 3065-3071.

72 Venter J.C., Adams M.D., Myers E.W., Li P.W., Mural R.J., *et al*. The sequence of the human genome. *Science*. 291(5507): 1304-1351.

73 Von Bernhardi R., de Ioannes A.E., Blanco L.P., Herrera E., Bustos-Obregon E. & Vigil P. 1990. Round-headed spermatozoa: a model to study the role of the acrosome in early events of gamete interaction. *Andrologia.* 22(1): 12-20.

74 Voullaire L., Wilton L., McBain J., Callaghan T. & Williamson R. 2002. Chromosome abnormalities identified by comparative genomic hybridization in embryos from women with repeated implantation failure. *Molecular Human Reproduction*. 8(11): 1035-1041.

75 Weenen C., Laven J.S., Von Bergh A.R., Cranfield M., Groome N.P., Visser J.A, Kramer P., Fauser B.C.J.M. & Themmen A.P.N. 2004. Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Molecular Human Reproduction*. 10(2): 77-83.

76 Yang Y.S., Rojas F.J. & Stone S.C. 1988. Acrosome reaction of human spermatozoa in zona-free hamster egg penetration test. *Fertility and Sterility*. 50(6): 954-959.

77 Younis J.S. 2003. Elevated progesterone: estradiol ratio—another test of ovarian reserve? *Fertility and Sterility*. 80(3): 679; author reply -80.

78 Zegers-Hochschild F., Adamson G.D., de Mouzon J., Ishihara O., Mansour R., Nygren K., Sullivan E. & Vanderpoel S. 2009. The international committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary on ART terminology. *Human Reproduction*. 24(11): 2683-2687.

79 Zegers-Hochschild F., Adamson G.D., de Mouzon J., Ishihara O., Mansour R., Nygren K., Sullivan E. & Vanderpoel S. 2009. The international committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary on ART terminology. *Human Reproduction*. 92(5): 1520-1524.

80 Zhang Y., Xu C.M., Zhu Y.M., Dong M.Y., Qian Y.L., Jin F. & **Huang H. 2007.** Preimplantation genetic diagnosis for Down syndrome pregnancy. *Zhejiang University-SCIENCE B*. 8(7): 515-521.