State of the Art of Assisted Human Reproduction

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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.
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
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
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 vitellic 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 (deleted, duplication, and translocation) and numerical (trisomy 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 phenotypical 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


