Chapter 1

Sperm Flow Cytometry: Beyond Human Fertilization and Embryo Development

Gerardo Barroso, Alexia Alvarez and Carlos Valdespin

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Abstract

Male infertily is a contributing factor in up to 50% of all infertility cases, a solo cause in about 30% of them. Therefore, new and improved diagnostic methods that reduce operator variability regarding sperm defects that are not accesible by the conventional microscope scoring should be evaluated. Assisted reproductive technology (ART) has been involved in the description of alternative pathways in basic cellular functions. it is important to know that it is also related to the peri-implantatory processes that involve the sperm-oocyte interaction, cellular changes observed during fertilization, and the early and late embryo development. Several pathways have been involved at the early stages of human gametogenesis. The spermatozoon has demonstrated an intricate correlation during the fertilization process, as a transfected vector on genetic material, and as interacting with other inner components (RNAm, mitochondrial organelles, etc.). Spermatogenesis is affected by programmed death cell pathways from its packaging process through the elongated cytoplasmic structures during spermiogenesis. Flow cytometry (FC) has been an outstanding tool with the capability to select human gametes to achieve a better reproductive condition. It has been applied as a diagnostic and therapeutic tool allowing a measurable and objective selection and discrimination of spermatozoa from subfertile subjects. Using FC, we are able to know that early distribution of organelles such as mitochondria has an impact in embryo quality before genetic activation on the eight-cell stages occurs. This chapter will let the readers know the current knowledge on sperm fertilization and the relation between the embryo development and the offspring and all the tools now available for an early diagnosis and to identify therapeutic options with FC.

Keywords: sperm, spermatozoa, flow cytometry, fertilization, embryo development, apoptosis, DNA fragmentation

1. Introduction

During the previous decades, the understanding of human reproductive processes has been newly addressed since the implementation of assisted reproductive technology (ART). In twenty-first century, novel data from cellular and molecular diagnostic techniques in the reproductive field play a primary role. ART has been involved in the description of alternative pathways in basic cellular functions not only in early reproductive processes as gametogenesis but also in peri-implantatory processes involving the sperm-oocyte interaction, cellular changes observed during fertilization and the early and late embryo development. The implementation of the flow cytometry (FC) in this field has contributed to the understanding of all of these processes as well as to the standardization of a growing number of both diagnostic techniques and therapeutic approaches.

Several processes have been involved at the early stages of human gametogenesis; the spermatozoon has demonstrated an intricate correlation during the fertilization process, not just as transfected vector on genetic material, but also in the interaction of other inner components (RNAm, mitochondrial organelles, etc.). Spermatogenesis is affected by programmed death cell pathways, from its packaging process through the elongated cytoplasmic structures during spermiogenesis. Flow cytometry has been applied as a diagnostic and therapeutic tool allowing a measurable and objective selection and discrimination of spermatozoa from subfertile subjects. Nowadays, the use of *in-vitro* fertilization including micromanipulation techniques as the intracytoplasmic sperm injection (ICSI) has demonstrated a better embryo outcome, applying the use of these discriminating tools.

The acrosome reaction is a primary step during the sperm/oocyte interaction where the content of several enzymes will be delivered into the egg cytoplasm. The scoring of acrosome-reacted human sperm allows a more accurate, faster, and simpler method using FC. The process of sperm chromatin decondensation occurs when a spermatozoon enters an ovum where the interaction between male and female pronuclei has shown to be prognostic during the early stages of embryo development. Today, by using FC we know that early distribution of organelles as mitochondrial has an impact on embryo quality before genetic activation on stages of eight cells occurs. Furthermore, polarity and blastocyst development may be affected by the egg/sperm condition during the peri-implantatory process.

The potential of producing offspring of a predetermined sex has been a goal that exercised the imagination of mankind over many generations. A desire to predetermine sex is generally based on social values or a wish to avoid the potential conception of a child with an X-linked recessive disorder. This is now possible by the selection of spermatozoa using FC.

The main objective of this chapter is to address the current knowledge on sperm fertilization and its relationship with the embryo development and the offspring, showing the diagnostic and therapeutic opportunities using FC as an accurate and objective tool. The chapter is divided in three main subjects: the fertilizing spermatozoa, fertilization assessment, and evidence from ART.

2. Flow cytometry to explore human spermatozoa: reproductive impact

During the last decades, adverse, male reproductive health condition has been associated with an increase of testicular cancer, hypospadias, anatomical dysfunction, and abnormal semen parameters, together with an increased demand for ART [1, 2]. Male infertility diagnosis includes a visual assessment of semen analysis referred by the World Health Organization guidelines [3], which is not standardized and variability has been demonstrated among technicians and laboratories worldwide [4, 5]. Additionally, this subjective evaluation of sperm morphology, viability, motility, and number does not evaluate the entire sperm biological characteristics that may play a critical role during the egg-spermatozoa interaction, and no molecular or functional tests are applied routinely. As male infertility contributes up to 50% of the dysfunction reproductive process [6], development of new and improved diagnostic methods that reduce operator variability reflecting sperm defects not accessible by the conventional microscope scoring [1], becomes critical. The use of FC in this field represents an automated, rapid, sensitive, objective, multiparametric, and reproducible approach for the assessment of male infertility, especially in candidates for ART [7]. Because a flow cytometer can acquire thousands of events in seconds at a fixed flow rate, it could be used to determine sperm concentration [8]. Assessing cell number with FC is frequently performed using DNA dyes to differentiate between haploid mature cells, haploid round spermatids and diploid cells, in order to determine the total number in a known volume while assessing spermatogenesis [7, 9]. Good flow adjustment and the use of fluorospheres as internal control improve the reliability of spermatozoa numbers [10].

Sperm motility has been evaluated by FC using mitochondrial membrane potential markers to test the effects of cell sorting, swim-up, cryopreservation, and attempts to create populations enriched in highly motile spermatozoa [11–13]. Studies of cell viability have used a wide variety of vital stains as propidium iodine (PI) and ethidium homodimer [7–9]. These dyes enter cells with a broken plasmalemma, emitting red florescence when binding to nucleic acids [8]. Other viability probes as fluorescein diacetate and SYBR-14 emit green fluorescence upon entering metabolically active cells. The combination of PI/SYBR-14 is probably the most widely used to evaluate sperm viability [8].

3. Acrosome reaction: fertilizing spermatozoa-oocyte binding

It is generally accepted that to fertilize the egg, ejaculated spermatozoa must undergo capacitation, recognize and bind to the zona pellucida, and undergo the acrosome reaction. The most significant changes experienced by sperm during capacitation are plasma membrane changes, increase in certain intracellular messengers, and increased phosphorylation of a set of proteins by different kinases [14, 15].

The acrosome reaction is an exocytic process of spermatozoa and an absolute requirement for fertilization [16]. Numerous sperm plasma membrane candidates for zona pellucida-sperm

binding have been described [17], but only some of them have been shown to be involved in the induction of the acrosome reaction. *In vivo*, the acrosome reaction is induced by a zona pellucida glycoprotein (ZP3), which may act as a ligand for one or more sperm plasma membrane receptor(s), while a second protein, ZP2, facilitates the secondary binding of acrosome-reacted spermatozoa to the zona matrix penetration [18]. Several studies have found that sperm responsiveness to acrosome reaction inducers was reduced in infertile patients [19, 20] therefore, evaluation of acrosome reaction can be used to predict fertilization success and can be a great help in choosing the most appropriate technique of ART [16]. Acrosome intactness has been traditionally examined by using phase-contrast, fluorescent, or even electron microscopy. However, the FC can be used instead of microscopy allowing determination of acrosome reaction and its temporal occurrence, and the examination of large sperm numbers [21]. The probes labeled with fluorescent agents bind to mannose and galactose moieties of the acrosomal matrix. As probes cannot penetrate an intact acrosomal membrane, only acrosome-reacted or damage spermatozoa will stain [22]. The double staining for membrane integrity and acrosomal integrity is relatively reliable for fresh and *in vitro* capacitated sperm.

Additionally, the potential of FC to study the molecular and physiological changes that render the sperm able to fertilize has been demonstrated in different studies. For example, the function of CD46 on human sperm inner acrosomal membranes is to protect the spermatozoa from complement-mediated lysis. Carver et al. demonstrated that it is feasible a high fertilization prediction by flow cytometric analysis of the CD46 antigen [23]. Moreover, there is a recent study that shows that the capacitation-associated hyperpolarization of the sperm plasma membrane potential involves a decrease in Na^+ mediated by inhibition of epithelial Na^+ channels, and regulated by PKA [24].

4. The ubiquitin-proteasome pathway

The ubiquitin is a protein present in all cells with a nucleus. This protein forms covalent bonds to other proteins with an isopeptide that binds the C-terminal glycine of ubiquitin and the E-amino group of a lysine in substrate proteins. This phenomenon is called ubiquitination. The ubiquitination has been related to several fertilization processes, for example, during spermatogenesis, there is a replacement in the spermatid's nuclear histones by transition proteins and permanent protamines [25]. This process is also present in the spermatid elongation with a drastic reduction of the sperm centrosome. The ubiquitination normally occurs in the cell cytosol or nucleus, but sometimes, when there is a defective spermatozoa, it occurs on their surface during posttesticular sperm maturation in the epididymus [26]. The detection of the surface-ubiquitin helps to identify defective spermatozoa and has the potential to become a biomarker for infertility diagnostics [1]. Detecting and measuring ubiquitin expression can be as easy as getting a single semen sample [26].

5. Human spermatozoa related to abnormal fertilization and embryo development: evidence from ART

In-vitro fertilization (IVF) facilitates the interaction between gametes, but beyond the resolution of fertility dysfunction, it has been shown its biological diagnostic qualities in the fertilization process. Intracytoplasmic sperm injection (ICSI) is a technique where the mature spermatozoon is injected into a metaphase II oocyte, it is regularly used in cases of male factor infertility. With this technique, the impact of the microinjection should be established to determine the safety of the process, and also the long-term possible consequences. This is because in ICSI many processes from natural fertilization are skipped.Although successful fertilization is unequivocally dependent upon multiple inherent qualities of the oocyte [27, 28], in the last two decades, several lines of evidence resulting from the use of ART provided initial support for the concept of paternal contribution to faulty fertilization and abnormal embryogenesis. The first days, the new zygote depends on the oocyte's endogenous information, initiating the cleavage divisions. Afterward, when the embryo is in a four to eight cell stage, the transcription starts. That is why the sperm nuclear defects are not regularly detected before an eight-cell stage embryo, time when a bigger expression of sperm genes started.

Sperm cytoplasm deficiencies can be detected in the single-cell zygote and then throughout the preimplantation development [29]. It is supposed that the early paternal effect may include sperm abnormalities related to activation of the oocyte (no sperm delivery or dysfunctional oocyte-activating factor) and aberrations of the centrosome-cytoskeletal device. Moreover, it is seen that the late paternal effect is connected to sperm alterations in the DNA chromatin and also probably in sperm mitochondrial or mRNAs delivery abnormalities.

The abnormalities related to genomic imprinting alterations can be presented in early and late paternal effects. Strong evidence associates the presence of abnormal sperm parameters (particularly teratozoospermia, but also oxidative damage and DNA fragmentation) with failed or delayed fertilization and, importantly, to aberrant embryo development.

Ejaculated human spermatozoa may present various degrees of DNA damage. Different theories have been proposed to explain its origin [30–32]:

(1) Harm could occur at the moment of DNA packing or can be the result of it during the transition of histone to protamine complex in toe spermiogenesis process; (2) the DNA fragmentation could be the consequence as well of direct oxidative damage that has been associated with xenobiotics, antioxidant depletion, smoking, heat exposure, presence of ions in sperm culture media, leukocyte contamination of semen, and (3) DNA damage that could be the consequence of apoptosis. The evidence of apoptosis in ejaculated spermatozoa could be the effect of several types of injuries [31, 32]. *In vivo*, apoptosis could be triggered at the testicular (hormonal depletion, irradiation, toxic agents, chemicals, and heat have been shown to induce apoptosis), epididymal (the result of signals released by abnormal and/or senescent spermatozoa or by leukocytes, such as reactive oxygen species and other mediators of inflammation/infection), or seminal (reactive oxygen species, lack of antioxidants, or other causes) levels. In addition, apoptosis could be triggered by factors present in the female tract.

In vitro, apoptosis is induced by incubation with inadequate culture media or different kind of manipulation procedures. Independently of the stimulus, spermatozoa that goes through apoptosis is not recognized by current methods. On the other hand, this spermatozoa could have the risk to transfer a damaged genome into the egg, and as a consequence having a poor embryo development, miscarriage, or birth defects [31].

For many years, it has been known that the chromatin of the mature sperm nucleus can be abnormally packaged [33]. In addition, abnormal chromatin packaging and nuclear DNA damage appear to be linked, and there is a strong association between the presence of nuclear DNA damage in the mature spermatozoa and poor semen parameters [31, 34, 35]. Endogenous basis in DNA are regularly expressed at particular stages of spermiogenesis in different animal species; these endogenous basis are present during spermiogenesis but they are not evident once chromatin packaging is completed. It is known that topoisomerase 11, an endogenous nuclease, forms and binds nicks to release torsional stress and fix chromatin rearrangement during protamination [36–38].

Several studies have demonstrated that sperm DNA quality has a robust power to predict fertilization *in vitro* [39–41]. Tomlinson et al. show that the only parameter that presented a significant difference between pregnant and nonpregnant groups in IVF was the amount of DNA fragmentation assessed by *in situ* nick translation.

The sperm chromatin structure assay (SCSA) has been considered as a diagnostic option to define fertilization by the evaluation of DNA stability. SCSA criterion is related to fertilization, blastocyst formation, and ongoing pregnancy in *in vitro* fertilization and ICSI cycles [42]. This assay measures susceptibility to DNA denaturation *in situ* in sperm exposed to acid for 30 s followed by acridine orange staining, and the use of FC in the SCSA increases its dependability [43, 44]. Moreover, evaluation of chromatin condensation with FC has demonstrated that swim-up and percoll gradient centrifugation methods improve the percentage of spermatozoa with normal chromatin structure in some samples with poor initial quality [45].

The expression of sperm apoptotic-like markers can be rapidly evaluated using FC and differences have been found in individuals with normal and abnormal sperm parameters [46–49]. One of the early steps of apoptosis is the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, which can be detected by sperm annexin V labeling. Currently, annexin V is available conjugated with a wide variety of fluorochromes to perform sperm FC analysis. Increased membrane permeability is another indicator of apoptosis and there are several dyes that are combined to identify membrane-undamaged spermatozoa with increased membrane permeability. The combination of annexin V with PI permits the identification of apoptosis-like changes and spermatozoa with compromised plasma membrane. This two parameters identify four categories of sperm: live, live early "apoptotic", dead and late "apoptotic", and late "necrotic" cells using FC [21]. Flow cytometric analysis of DNA fragmentation evaluated by terminal deoxynuceotidyl transferase-mediated UTD nick-end labeling (TUNEL) measures changes at the later stage (late apoptosis), while annexin V/PI measure early sperm apoptosis-like changes [21].

Caspases are the central components in the apoptosis signaling cascade. Members of this family of cysteine proteases have been found in their inactive and active states in spermatozoa [50]. The detection of activated caspases in living spermatozoa can be performed by using fluorescence labeled inhibitors of caspases, which are cell permeable and noncytotoxic [50]. Active caspases will form a covalent bond with the reagent, so retain the fluorescent signal within the cell and can be easily detected by FC. Any unbound inhibitor leaves the sperm [51].

6. Gender DNA-based spermatozoa sex selection

Evidence of regular methods of preconceptional gender selection show that *in vivo* methods such as timed intercourse, ovulation induction, and artificial insemination do not affect the sex ratio in a clinically significant percentage. *In vitro* separation of X- and Y-bearing spermatozoa by gradient methods change significantly the sex ratio at birth. Nevertheless, these trials were non controlled, and molecular biological techniques were not able to prove that these methods changed the Y- to X-bearing spermatozoa ratio significantly for clinical use. However, recent scientific studies have made reliable preconceptional sex selection possible by using preimplantation diagnosis or sperm separation by FC combined with IVF. Actually, these methods are used to avoid sex-linked disorders. The two of them include in vitro fertilization as an invasive procedure and most of them do not have a medical and therapeutical indication. Both involve the invasive procedure of IVF and thus are held by most as inappropriate for nonmedical indications. The improvement in FC output of selected X and Y spermatozoa could bring in the future sufficient selected gametes for artificial insemination. This technique will provide a viable noninvasive technique for the selection of sex for social purposes.

FC for gender selection is based on the variability of chromatin staining by DNA-binding fluorescent dye Hoechst 33342, detecting the fluorescence from individual cells, and classifying individual cells based on fluorescence. Afterward, selected spermatozoa are used for based on polymerase chain reaction (PCR) or FISH on treated spermatozoa, or by biopsy of the developing embryo (preimplantation genetic diagnosis, PGD).

Main concerns regarding safety of sorting technique are the possible hazards imposed especially on the DNA of the spermatozoa. A chemical compound is used that binds to DNA and emits energy in form of light when hit by a laser beam. Also the laser beam transmit energy to the atoms inside and around DNA. This excessive energy with the combination of many chemical compounds, and water, may create molecular species that can break other molecular junctions and cause damages in DNA. In the mature sperm head, there are no mechanisms to fix the damaged DNA; this situation only takes place in the egg after fertilization. The higher the number of disruptions of the DNA, the higher number of the error probabilities.

In 1998, Fugger et al. presented births of normal female after sperm sorting techniques and subsequent IUI, IVF, or ICSI. Indications were sex-linked disorders or family balancing. From 27 patients that were treated in 33 cycles with X-sorted spermatozoa, seven pregnancies were after IVF or ICSI treatment. IUI was performed in 208 cycles in 92 patients. The results were 22 clinical pregnancies, of which seven ended in spontaneous miscarriage, one of them was an ectopic pregnancy, and 12 of them were still ongoing when the study was published. Nine of

the pregnancies terminated in eleven healthy children. In 17 cases where the sex was known at the time of publication 15 were female. In a subsequent report on 332 patients, 96 pregnancies were achieved in 663 cycles; desired gender was obtained in 94% (37/39) of cases for parents desiring females and in 73% (11/15) of cases for those desiring males. At publication, 47 pregnancies were ongoing.

7. Perspectives for sperm flow cytometry

In the last two decades, FC has revolutionized the perspective of reproductive biology. Several dogmas have changed not just in the understanding of sperm-oocyte interaction, but mainly in the fertilization process. Assisted reproductive technology has provided to be efficient helping infertile couples and to raise this reproductive goals, we have to understand the molecular network related to the human model. Flow cytometry has been an outstanding tool with the capability to select human gametes to achieve a better reproductive condition. Moreover, FC has made clear these processes under the molecular understanding, but primarily for the clinical application. Human fertilization and embryo development are a wide field where for the next years, we will be witnesses of the offspring modulation and the FC will still being an outstanding resource.

Author details

Gerardo Barroso[1,2*], Alexia Alvarez[3,2] and Carlos Valdespin[3,2]

*Address all correspondence to: barrosog@me.com

1 Medical Director, Center for Reproduction and Clinical Research, Nascere, Mexico City, Mexico

2 Obstetrics and Gynecology Department, American British Cowdray Hospital, Mexico City, Mexico

3 Medical Associated, Center for Reproduction and Clinical Research, Nascere, Mexico City, Mexico

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