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Developmental sperm contributions: fertilization and beyond

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The objective of this review was to examine the role of the various spermatozoal components suspected of actively participating in early human development. The contributions of the fertilizing spermatozoon to the oocyte include, as a minimum, the delivery of the DNA/chromatin, a putative oocyte-activating factor (OAF), and a centriole. Recent data indicate that spermatozoa may also provide the zygote with a unique suite of paternal mRNAs; some transcripts might be crucial for early and late embryonic development and deficient delivery, or aberrant transcription might contribute to abnormal development and arrest. Clinical evidence from assisted reproduction suggests that failure to complete the fertilization process, syngamy, or early cleavage might be the result of an early paternal effect. It is speculated that an abnormal release of a putative OAF and/or dysfunctions of the centrosome and cytoskeletal apparatus may mediate these effects. On the other hand, a later paternal effect resulting in embryonic failure to achieve implantation, pregnancy loss, and/or developmental abnormalities resulting from "carried over" sublethal effects may be associated with sperm nuclear/chromatin defects, including the presence of aneuploidy, genetic anomalies, DNA damage, and possibly other causes. These findings highlight the need for continuous monitoring of clinical results. (Fertil Steril® 2009;92:835–48. ©2009 by American Society for Reproductive Medicine.)

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Past research has viewed the spermatozoon as a simple carrier or vector that transfers DNA to the egg, its contribution to embryogenesis being crucial for delivering the genetic material. It is now well established that there is extensive cross-talk between the fertilizing sperm and the egg, leading to activation of the egg on one hand and sperm head decondensation on the other. This is orderly followed by female and male pronuclear formation, syngamy, and the first cleavage divisions. Several structures/organelles and molecules present in the spermatozoon appear to be critical for the accomplishment of the milestones resulting in normal fertilization and early embryo development.

The fertilizing human spermatozoon is essential for contributing at least three components: [1] the paternal haploid genome, [2] the signal to initiate metabolic activation of the oocyte, and [3] the centriole, which directs microtubule assembly leading to the formation of the mitotic spindles during the initial zygote development. Clinical evidence derived from the use of assisted reproductive technology (ART) points to the fact that defective sperm contributions may extend beyond fertilization, highlighting the fact that early and late paternal effects may be determinants of abnormal development.

Intracytoplasmic sperm injection (ICSI) is a relatively new ART that involves injection of a mature spermatozoon into a metaphase II oocyte, typically performed in cases of male factor infertility. As such, this technique bypasses multiple steps of the natural fertilization process by introducing an apparently intact spermatozoon into the ooplasm. The impact of the microinjection technique on fertilization and postfertilization events should be definitely established to determine the immediate safety of ART as well as any possible long-term consequences, including embryonic anomalies that result in developmental arrest or are carried over to the offspring.

The objective of this review was to examine the role of the various spermatozoal components/molecules anticipated to participate in early human development. To accomplish this goal, we first summarize recent knowledge on the biology of sperm-egg interaction, focusing on data derived from both human and nonhuman models, and then discuss clinical evidence derived from the ART setting supportive of crucial contributions of the male gamete during early and late embryo development.

BIOLOGICAL CONSIDERATIONS

Sperm-Oocyte Interaction

It is generally accepted that to fertilize the egg, ejaculated spermatozoa must undergo capacitation, recognize and bind to the zona pellucida (ZP), and undergo the acrosome reaction (AR; Figs. 1 and 2)

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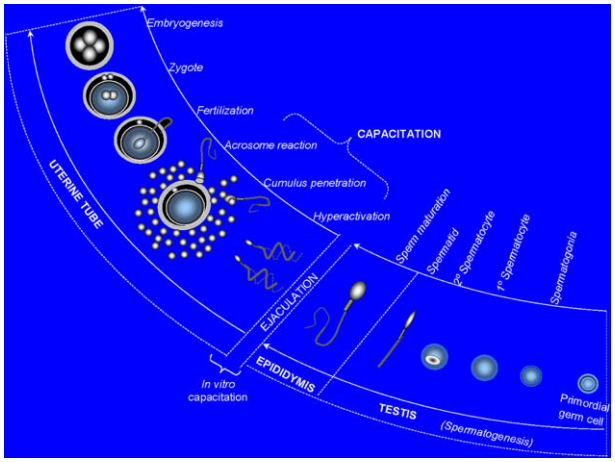
FIGURE 1

Schematic of the journey of the sperm cell during spermatogenesis and transit through the male and female tracts (modified from reference 1, with permission).



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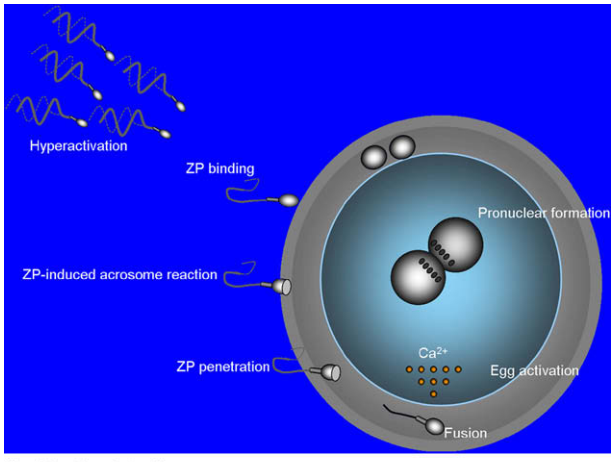
(1). The most significant changes experienced by sperm during capacitation are plasma membrane changes, increase in certain intracellular messengers, and increased phosphorylation of a set of proteins by different kinases (2–4).

In the murine species, a well-characterized model of gamete interaction, tight gamete binding is probably achieved through interaction of the ZP protein 3 (ZP3) and yet unidentified complementary sperm-binding protein(s) present in the plasma membrane. It is agreed that ZP3 triggers the AR that is then followed by a secondary binding process involving the ZP protein-2 (ZP2) and the inner acrosomal sperm membrane leading to zona penetration. Glycosylation appears mandatory for murine ZP3-ligand function. It has been demonstrated that O-glycosylation, and particularly terminal galactose residues of O-linked oligosaccharides, are essential for maintaining mouse gamete interaction. Others have provided evidence that the amino sugar N-acetylglucosamine is the key terminal monosaccharide involved in sperm-zona interaction in the mouse. In contrast, AR-triggering activity of ZP3 seems to depend upon the integrity of the protein backbone (5–7).

FIGURE 2

Critical steps involved in sperm-oocyte interaction leading to pronuclear formation and syngamy.



Note: ZP = Zona Pellucida.

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For the last two decades, investigators have sought to identify an individual protein or carbohydrate side chain as the "sperm receptor." In earlier work performed in nonhuman models, diverse candidates were postulated as primary sperm receptors for ZP3: [1] a 95 kd tyrosine-kinase (8); [2] sp56 (9); [3] trypsine-like protein (10); [4] β1-4 galactosyltransferase (11); and [5] spermadhesins (12). However, none of these molecules has been unequivocally established as an active receptor, and the physiological relevance of these candidates is still under debate.

In more recent experiments performed in knockout mice with absence of either ZP2 or ZP3 expression, it was demonstrated that the ZP fails to assemble around growing oocytes and that females are infertile; on the other hand, in the absence of ZP1 expression, a disorganized zona assembles around growing oocytes and females exhibit reduced fertility. These and other observations led to the speculation of a model for ZP structure in which ZP2 and ZP3 form long Z-filaments cross-linked by ZP1 (13).

The identity of the sperm-binding proteins remains a subject of active investigation, and the most recent data indicate that many different proteins are likely involved. There has been recent progress in functionally characterizing two promising candidate proteins that interact with ZP3: zonadhesin and PKDREJ; further, evolutionary analysis of both proteins within and between species of primates has identified promising regions of the proteins that may interact and coevolve with ZP3 (14–16).

In recently published studies, a group of investigators isolated, purified, and solubilized plasma membranes of porcine sperm heads (17). Three persistently bound proteins were identified by tandem mass spectrometry as isoforms of AQN-3, probably representing the main sperm protein involved in ZP binding in this species. This protein is a member of the spermadhesin family, a group of secretory proteins expressed in the epididymis. P47, fertilin beta, and peroxiredoxin 5 were also conclusively identified. More studies are needed to validate and characterize these proteins in other species.

Previous reports suggested that murine ZP2 mediates secondary binding of spermatozoa and that cleavage of ZP2 by proteases released through cortical granule reaction causes zona "hardening" and thus prevents polyspermy. Using an elegant approach, an observed postfertilization persistence of mouse sperm binding to "humanized" ZP was shown to correlate with uncleaved ZP2. These observations are consistent with a model for sperm binding in which the supramolecular structure of the ZP necessary for sperm binding is modulated by the cleavage status of ZP2 (18–21). To test the alternative hypothesis that the protein sequence of murine ZP glycoproteins mediates sperm-egg binding, transgenic mice were created in which mouse ZP3 was replaced with human ZP3. If initial gamete binding were protein mediated, such mice would bind human sperm. However, murine but not human sperm were found to bind to mouse eggs expressing human ZP3 (18).

During fertilization, the acrosome-reacted spermatozoa fuse with the oolema, and the whole spermatozoon (with the exception of part of the sperm membrane, most of the outer acrosomal membrane, and the acrosomal components) is incorporated into the oocyte (22). The

ZP binds to at least two different receptors in the sperm head plasma membrane. One is a G_i-coupled receptor that can activate phospolipase Cβ1 and regulates adenylyl cyclase to increase cyclic adenosine monophosphate (cAMP) levels. The cAMP activates protein kinase A (PKA) to open a calcium channel in the outer acrosomal membrane, resulting in a relatively small rise in cytosolic Ca^{2+}. Calcium activates phospholipase Cγ, which is coupled to the second tyrosine kinase receptor. The products of phospholipase C activity, diacylglycerol and inositol triphosphate (IP3), lead to activation of protein kinase C (PKC) and IP3 receptor; PKC opens a calcium channel in the membrane, and IP3 activates the calcium channel in the outer acrosomal membrane leading to a higher increase in cytosol calcium, which results in membrane fusion and completion of the AR (23–25). Results of recent studies indicate that components that are essential for intracellular membrane fusion in somatic cells, such as Rab3A, GTPase, and SNAREs, may be present in mammalian sperm and may also participate in membrane fusion during the AR (26).

A New Molecular Model for Human Sperm-Egg Interaction

Compelling evidence has now demonstrated that carbohydrate-binding proteins on the sperm surface mediate gamete recognition by binding with high affinity and specificity to complex glycoconjugates of the ZP (27–32). A group of investigators has recently reported a series of studies that suggest that [1] the binding protein(s) on human spermatozoa recognize selectin ligands or molecules alike on the ZP to ensure recognition and attachment; and [2] the human ZP expresses glycans structurally (and probably functionally) linked to natural killer cell inhibition (30, 32–36).

Those results are strengthened by the discovery that glycodelyn-A, an endometrial epithelial protein, produced a potent and dose-dependent inhibition of sperm-zona binding under in vitro conditions (32, 37, 38). This turned out not to be surprising since it was found that the oligosaccharides associated with this glycoprotein (fucosylated lacdiNAc antennae among others) also potently block selectin-mediated adhesions. Results also indicated the possibility that the carbohydrate binding specificity of the receptors mediating gamete recognition and lymphocyte/leukocyte adhesion have converged, at least to some extent. This concept is further supported by the demonstration of contraceptive and immunosuppressive properties of glycodelin-A and their carbohydrate dependence (30, 39, 40). Three major classes of N-glycans were detected, [1] high mannose, [2] biantennary bisecting type, and [3]) biantennary, triantennary, and tetra-antennary oligosaccharides terminated with $Lewis^x$ and $Lewis^y$ sequences (39–41). Thus, the major N-glycans of human sperm are associated with the inhibition of both innate and adaptive immune responses. These results provide more support for the eutherian "fetoembryonic defense system hypothesis" that links the expression of carbohydrate functional groups to the protection of gametes and the developing human in utero (30, 31).

Based on the previous data showing that glycodelin-A receptor(s) and ZP protein receptor(s) on human spermatozoa are closely related, Chiu et al. (42) used a chemical cross-linking approach to isolate the glycodelin-A sperm receptor complex from human spermatozoa. The receptor was identified to be fucosyltransferase-5 (FUT5) by mass spectrometry and confirmed with the use of anti-FUT5 antibodies. Biologically active FUT5 was purified from spermatozoa and bound strongly to intact and solubilized human ZP. These observations provided strong evidence that human sperm FUT5 is a receptor of glycodelin-A and ZP proteins and that glycodelin-A inhibits spermatozoa-zona binding by blocking the binding of sperm FUT5 to the ZP.

In the human, native solubilized ZP triggers the AR. Cross et al. (43) were the first to report that treatment of human sperm in suspension with acid-disaggregated human ZP (2–4 ZP/μL) increased the incidence of acrosome-reacted sperm. Lee, Check, and Kopf (44) demonstrated that pertussis toxin treatment of human sperm inhibits the (solubilized) ZP-induced AR. In contrast, acrosomal exocytosis induced by the calcium ionophore A-23187 is not inhibited by pertussis toxin pretreatment. Studies by Franken, Morales, and Habenicht (45) showed a dose-dependent effect of solubilized human ZP on the AR in the range of 0.25–1 ZP/μL and also confirmed the involvement of G_i protein during ZP-induced AR of human sperm. Schuffner and colleagues (46) reported that [1] acrosomal exocytosis of capacitated human sperm triggered by the homologous ZP is dependent on the activation of G(i) proteins (pertussis toxin sensitive) and the presence of extracellular calcium; and [2] P and follicular fluid exert a priming effect on the ZP-induced AR.

Recent studies have revealed that human ZP is comprised of four glycoproteins designated as human ZP1, ZP2, ZP3, and ZP4 (47, 48). The ortholog of the human *ZP4* gene is present in the mouse genome as a pseudogene. Chiu et al. (49) investigated the effects of native human ZP3 and ZP4 on AR and spermatozoa-ZP binding. Native human ZP3 and ZP4 were immunoaffinity purified. The investigators induced AR and inhibited spermatozoa-ZP binding time and dose dependently to different extents. These biological activities of human ZP3 and ZP4 depended partly on their glycosylation, with N-linked glycosylation contribution being more significant than O-linked glycosylation. Although progress is being made, the specific contributions of sperm-egg receptors/ligands involved in ZP binding are far from being resolved.

Sperm-Oocyte Fusion

Sperm-oocyte fusion is a cell-cell membrane fusion event. The inner and outer acrosomal membranes and the plasma membrane of the equatorial region remain intact after the completion of the AR and zona penetration (50, 51). Acrosome-reacted sperm bind to and fuse with the egg plasma membrane at the postacrosomal region of the sperm; this region is capable of fusion only after acrosomal exocytosis has taken place (28). Several candidate binding molecules have been reported.

Binding of sperm to the egg plasma membrane appears to be mediated by members of the cysteine-rich secretory protein family (CRISP1 and CRISP2), a member of the ADAM family of transmembrane proteins on sperm, and the integrin $alpha_{vi}ß_1$ receptor on eggs (52). Sperm binding to an egg integrin (ß1) is a prerequisite adhesion step for sperm-egg membrane fusion in mammalian fertilization (53). The oocyte integrin is required for membrane fusion, and its activity appears to be related to a sperm surface protein fertilin (termed PH-30) that was implicated in gamete fusion based on antibody inhibition studies. Although some ADAM proteins act to block or to promote protease activity, fertilin has no such roles (54).

P-selectin is expressed on the oolemma of human and hamster oocytes after sperm adhesion and is also detected on the equatorial region of acrosome-reacted human spermatozoa, suggesting that this selectin might be involved in gamete interaction (55). In addition, epididymal protein DE or CRISP1 and testicular protein Tpx-1 also known as CRISP2 are cysteine-rich secretory proteins that are also apparently involved in gamete fusion through interaction with egg-binding sites (56). Other candidates have been proposed, including equatorin and CD9 (57–59). Inoue et al. (60)

identified a mouse sperm fusion-related antigen and showed that the antigen belongs to a novel immunoglobulin superfamily protein. The investigators termed the gene Izumo and produced a gene-disrupted mouse line. Izumo2/2 mice were healthy, but males were sterile. They produced normal-looking sperm that bound to and penetrated the ZP but were incapable of fusing with eggs. Human sperm also contain Izumo, and addition of the antibody against human Izumo left the sperm unable to fuse with zona-free hamster eggs. However, glycosylation appears not to be essential for the function of Izumo (61). The specific roles of all these molecules need to be further validated (62).

Oocyte Activation

The signaling mechanism used by the spermatozoa to initiate and perpetuate oocyte responses is unclear, and three theories have been proposed: [1] the fusion theory, which suggests the presence of active calcium-releasing components in the sperm head (63, 64); [2] the receptor theory, which proposes a receptor-mediated signal transduction localized on the oocyte plasma membrane (65); and [3] the "calcium bomb" theory, which proposes that upon fertilization Ca^{2+} enters the egg either from stores in the sperm itself or through channels in the sperm's plasma membrane (66).

It was earlier reported that a cystosolic sperm factor containing a protein called oscillin, which is related to a prokaryote glucosamine phosphate deaminase and is located in the equatorial segment, appeared to be responsible for causing the calcium oscillations that trigger egg activation at fertilization in mammals (67). However, experimental evidence has now shown that oscillin is not responsible for the mammalian sperm calcium oscillations (68).

Present evidence supports the concept that an inositol-3-phosphate (IP3) receptor system is the main mediator of calcium oscillations in oocytes (67). It has recently been shown that the soluble sperm factor that triggers calcium oscillations and egg activation in mammals is a novel form of phospholipase C (PLC) referred to as PLC zeta (69). This has been demonstrated by injection into eggs of both c-RNA encoding for PLC zeta and recombinant PLC zeta (69, 70). According to a present hypothesis, after fusion of the sperm and egg plasma membrane, the sperm-derived PLC zeta protein diffuses into the egg cytoplasm, giving as a result the hydrolysis of PIP2 (phosphatidylinositol 4, 5-bisphosphate) from an unknown source to generate IP3 (inositol 1,4,5-trisphosphate) (71, 72).

The earliest visible indications of the transition of mammalian eggs, or egg activation, are cortical granule extrusion (CGE) by exocytosis and resumption of meiosis. Although these events are triggered by calcium oscillations, the pathways leading to the intracellular calcium release are not completely understood. The Ca^{2+} transients stimulate the resumption of the cell cycle by decreasing the activity of both an M-phase-promoting factor and a cytostatic factor (73) and either the Ca^{2+} transients and/or PKC lead to CGE (74). Therefore, the calcium transients and/or activation of PLC zeta lead to CGE by yet an undefined mechanism. Src family kinases (SFK) have been recently suggested as possibles inducers of some aspects of egg activation, although a role for SFK upstream of calcium release remains plausible (75).

Recently, two sperm-borne proteins that induce formation of pronuclei in eggs have been described: [1] the truncated c-Kit tyrosine kinase, which activates the dormant egg by eliciting intracellular Ca^{2+} oscillations, which serve as a secondary messenger for downstream effectors of zygotic development (76, 77); and [2] the protein PAWP (postacrosomal sheath WW domain binding protein). PAWP exclusively resides in the postacrosomal sheath of the sperm perinuclear theca (PT). Microinjection of recombinant PAWP or alkaline PT extract into metaphase-II-arrested porcine, bovine, macaque, and xenopus oocytes induced a high rate of pronuclear formation, which was prevented by coinjection of a competitive peptide derived from PAWP but not by coinjection of the point-mutated peptide. ICSI of porcine oocytes combined with coinjection of the competitive peptide or an antirecombinant PAWP antiserum prevented pronuclear formation and arrested fertilization (78).

Fate of Sperm Mitochondrial DNA

Most cells in the body contain between 103 and 104 copies of mitochondrial DNA (mtDNA). However, there are slightly higher copy numbers (n = 106) in mature oocytes. This may be in preparation for the energetic demands of embryogenesis (79–81). Spermatozoa, on the other hand, are metabolically flexible and, in some species, can switch between aerobic and anaerobic metabolism, which reflects the great range of oxygen tensions they experience, from near anoxia in the testis and epididymis, to ambient tension in the vagina environment and in vitro. Like somatic mitochondrial DNA (mtDNA), that of spermatozoa is highly vulnerable to mutation and a significant number of mtDNA deletions are found in the semen of at least 50% of normospermic men (82, 83).

Given the lengthy process of spermiogenesis and epididymal maturation during which the sperm and mitochondria have to survive, the likelihood of being exposed to mutagenic agents is high. Indeed, the need to exclude defective sperm mtDNA from contributing to the embryo is possibly one of the major selection pressures against survival of paternal mtDNA. Short (84) has suggested that the asymmetric inheritance of mtDNA may be the fundamental driving force behind amphimixis and anisogamy because of the need to conserve a healthy stock of mtDNA for embryo development through a long period of quiescence in meiosis.

The strictly maternal inheritance of mtDNA in mammals is a developmental paradox because the fertilizing spermatozoon introduces up to 100 functional mitochondria into the oocyte cytoplasm at fertilization (85). However, destruction of sperm mitochondria appears to be an evolutionary and developmental advantage (86) because the paternal mitochondria and their DNA may be compromised by the deleterious action of reactive oxygen species (ROS) encountered by the sperm during spermatogenesis, storage, migration, and fertilization (87). Studies have shown that the mitochondrial membrane proteins, rather than mtDNA, seem to determine whether the sperm mitochondria and mtDNA are passed on or degraded (88).

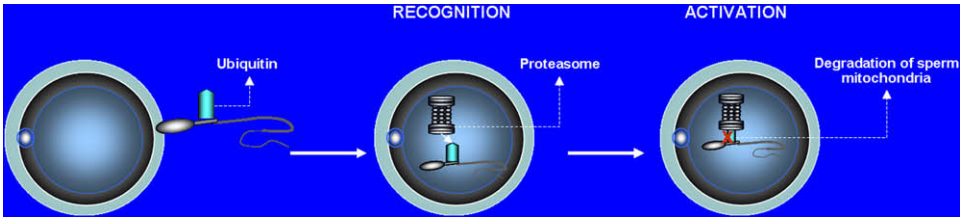
The Ubiquitin-Proteasome Pathway

Ubiquitin, a highly conserved protein present in apparently all eukaryotic cells, has the property of binding covalently to other proteins, via an isopeptide bond between the C-terminal glycine of ubiquitin and the E-amino group of a lysine in substrate proteins, a process called ubiquitination. The post-translational modification by ubiquitination marks defective or outlived intracellular proteins for proteolytic degradation by the 26S proteosome or by lysosomes (89). One of the most significant processes related to the ubiquitin-proteasome pathway in fertilization is the destruction of sperm mitochondria (90, 91) (Figure 3).

The true identity of ubiquinated substrates in the sperm mitochondria is not known. Nevertheless, it was recently shown that prohibitin, a mitochondrial membrane protein, is one of the ubiquitinated substrates that makes the sperm mitochondria responsible for the egg's ubiquitin-proteasome-dependent proteolytic machinery

FIGURE 3

Scheme of the ubiquitin-proteasome pathway in fertilization.



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after fertilization (92) Abnormalities of this recognition system might be involved in the deregulation of mitochondrial inheritance and sperm quality control.

Ubiquitin tagging of sperm mitochondrial membranes is not the only ubiquitin-proteasome pathway related to fertilization. During spermatogenesis, the ubiquitination process is indispensable in the replacement of the spermatid's nuclear histones by transition proteins, followed by permanent substitution with protamines (93, 94). Ubiquitination also has a principal role in the dramatic reduction of human sperm centrosome that occurs during spermatid elongation. Protein ubiquitination typically occurs in the cell cytosol or nucleus, and defective mammalian spermatozoa become ubiquitinated on their surface during post-testicular sperm maturation in the epididymus (95). Moreover, it has recently been postulated that sperm-acrosomal ubiquitin C-terminal hydrolases are involved in sperm-ZP interactions and antipolyspermy defense (96).

Morphologically normal and abnormal ubiquitinated sperm are found in the ejaculate of mammals (97, 98). The latter have apparently escaped from epididymal degradation and are characterized by main defects of head and/or axoneme (99). Sutovsky et al. (100, 101), found a discrete association of bovine semen ubiquitination with DNA fragmentation and suggested that sperm ubiquitination is associated with poor-quality sperm parameters in men. However, other studies have challenged this finding (102).

Pronuclear Interaction and Nuclear Fusion

In primates, the male pronucleus is tightly associated with the centrosome, which nucleates microtubules to form the sperm aster whose growth drives the centrosome and associated male pronucleus from the cell cortex toward the center of the oocyte. Structural abnormalities or incomplete junctioning of the centrosome have been identified as a novel form of infertility (103). In contrast, the female pronucleus has neither an associated centrosome nor microtubule nucleating activity. Nevertheless, the female pronucleus moves along microtubules from the cell cortex toward the centrosome located in the center of the sperm aster. The current model for the movement of the female pronucleus involves its translocation along the microtubule lattice using the minus-end-directed motor dynein in a manner analogous to organelle motility (104–106).

Mammalian fertilization requires dynein and dynactin to mediate genomic union; dynein concentrates exclusively around the female pronucleus, whereas dynactin localizes around the pronuclei and associates with nucleoporins and vimentin, in addition to dynein (107, 108). The findings that a sperm aster is required for dynein to localize to the female pronucleus and that the microtubules are necessary to retain dynein, but not dynactin, at their surface suggest that nucleoporins, vimentin, and dynactin might associate upon pronuclear formation and that subsequent sperm aster contact with the female pronuclear surface allows dynein to interact with these proteins (106–108).

EVIDENCE FOR PATERNAL CONTRIBUTIONS TO ABNORMAL FERTILIZATION AND EMBRYOGENESIS

Clinical Evidence: Lessons from the IVF/ICSI Setting

Successful fertilization is unequivocally dependent upon multiple inherent qualities of the oocyte (109, 110). 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. Strong evidence associated 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. These results are summarized in Table 1 (111–130).

The newly formed zygote undergoes early cleavage divisions depending upon the oocyte's endogenous machinery. Transcription is initiated at the 4- to 8-cell stage of the embryonic genome (131). Consequently, sperm nuclear deficiencies are usually not detected before the 8-cell stage, when a major expression of sperm-derived genes has begun. On the other hand, sperm cytoplasm deficiencies can be detected as early as the 1-cell zygote and then throughout the preimplantation development (127).

The terms "late" and "early" paternal effects have been proposed to denote these two pathological conditions (127, 132). The diagnosis of an early paternal effect is based upon poor zygote and early embryo morphology and low cleavage speed, and it is not associated with sperm DNA fragmentation. The late paternal effect, on the other hand, is manifested by poor developmental competence leading to failure of implantation and is associated with an increased incidence of sperm DNA fragmentation in the absence of zygote and early cleavage stage morphological abnormalities. It has been suggested that ICSI with testicular sperm can be an efficient treatment for the late paternal effect (133).

It can be speculated that the early paternal effect may include sperm dysfunctions related to oocyte activation (no sperm delivery of or dysfunctional oocyte-activating factor [OAF]) and aberrations of the centrosome-cytoskeletal apparatus. On the other hand, the late paternal effect is associated with sperm abnormalities at the level of DNA chromatin and perhaps sperm mitochondrial dysfunctions or abnormal sperm mRNAs delivery (see below). Alterations due to genomic imprinting anomalies probably result in both early and late paternal effects.

TABLE 1

Several lines of evidence resulting from the use of ART provide support for the concept of paternal contributions to faulty fertilization and abnormal embryogenesis.

Authors	Evidence
Kruger et al. 1988 (111); Oehninger et al. 1988 (112); Oehninger et al. 1988 (113); Oehninger et al. 1989 (114)	Abnormal sperm parameters, particularly teratozoospermia, are associated with fertilization disorders in IVF, including failure and delayed fertilization.
Ron-el et al. 1991 (115); Parinaud et al. 1993 (116)	Abnormal sperm parameters associated with embryo cleavage deficiencies.
Grow et al. 1994 (117); Oehninger et al. 1996 (118); Mercan et al. 1998 (119)	Although multiple studies have shown that the outcome of clinical pregnancies after ICSI is not affected by semen quality, patients with severe teratozoospermia demonstrated a low implantation rate.
Gorczyca et al. 1993 (120); Hughes et al. 1996 (121); Lopes et al. 1998 (122); Duran et al. 2002 (123); Liu et al. 2004 (124)	Spermatozoa from infertile subjects contain various nuclear alterations (abnormal chromatin structure, chromosomal abnormalities, microdeletions of Y chromosome, and DNA strand breaks).
Barroso et al. 2000 (125); Aitken et al. 2001 (126); Tesarik et al. 2004 (127); Barroso et al. 2006 (128)	Poor sperm quality is associated with increased sperm aneuploidy and DNA damage (fragmentation, instability, and single-stranded DNA).
Jakab et al. 2003 (129); Bartoov et al. 2005 (130)	Other sperm abnormalities have also been associated with failed fertilization and aberrant or arrested embryo development; studies suggest that abnormal sperm might be excluded using novel technologies.

Note: IVF = In vitro fertilization; ICSI = Intracytoplasmic sperm injection; DNA = Deoxyribonucleic acid.

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Disorders of Oocyte Activation, Centrosome-Cytoskeletal Apparatus Dysfunction, and Mitochondria Elimination

There are clinical situations that might be explained by absence or dysfunction of the OAF. For example, it has been suggested that up to 40% of failed fertilization cases after ICSI could be due to the failure of the egg to activate (134). In these cases, the sperm is within the cytoplasm but a stimulus for activation is apparently missing. Certainly, there could be other cases in which the sperm provides the OAF but any of the multiple elements of the oocyte responsive system (SFKs, PIP2, 1P3 receptor, or PKC) could be aberrant, resulting in failure to resume meiosis or to undergo CGE.

It has recently been reported that sperm from a group of patients who repeatedly failed ICSI had undetectable PLC zeta and were unable to induce $Ca2^{+}$ oscillations in mouse eggs (135). Using mouse eggs, the investigators rescued egg activation by injection of mouse PLC zeta mRNA, indicating that the inability of human sperm to initiate $Ca2^{+}$ oscillations lead to failure of egg activation. Such results provide strong evidence for an abnormal PLC zeta expression underlying this functional defect.

Dysfunctional microtubule organization in failed fertilization during human IVF suggested that centrosomal dysfunction might be a cause of fertilization arrest. Microtubules and DNA were imaged in inseminated human oocytes that had been discarded as unfertilized (136). Results showed that fertilization arrested at various levels: [1] metaphase II arrest, [2] arrest after the successful incorporation of the spermatozoon, [3] arrest after the formation of the sperm aster, [4] arrest during mitotic cell cycle progression, and [5] arrest during meiotic cell cycle progression. In another study, it was demonstrated by inmunofluorescence analysis that a main reason of fertilization failure after IVF was no sperm penetration (55.5%). On the other hand, fertilization failure after ICSI was mainly associated with incomplete oocyte activation (39.9%) (134).

In a nonhuman primate model using apparently normal gametes, ICSI resulted in abnormal nuclear remodeling during sperm decondensation due to the presence of the sperm acrosome and apical perinuclear theca, structures normally removed at the oolema during IVF; this in turn caused a delay of DNA synthesis (137). Such unusual modifications brought concerns about the normalcy of the fertilization process and cell cycle checkpoints during ICSI (138).

The sperm acrosome contains a variety of hydrolytic enzymes that release into the ooplasm and might generate some kind of damage (139). It is unclear how an oocyte that has been microinjected with an acrosome-intact spermatozoon will cope with the sperm acrosome. It is believed that an acrosome introduced into the ooplasm by ICSI seems to disturb sperm chromatin decondensation physically. Katayama, Koshida, and Miyake (140) showed detailed morphological characteristics of the acrosome of boar sperm through ICSI, showing that the RNA-binding properties of sperm head components introduced into the cytoplasm were different from those after IVF. Resumption of meiosis and cortical- granules exocytosis was achieved after micromanipulation techniques.

Terada et al. (141) assessed the centrosomal function of human sperm using heterologous ICSI with rabbit eggs. They demonstrated that the sperm aster formation rate was lower in infertile men compared with in controls. Moreover, the sperm aster formation rate correlated with the embryonic cleavage rate after human IVF. The data suggested that reproductive success during the first cell cycle requires a functional sperm centrosome; dysfunctions of this organelle could be present in cases of unexplained infertility.

Kovacic and Vlaisavljevic (142) studied the microtubules and chromosomes of human oocytes failing to fertilize after ICSI. The results showed a high proportion of oocytes arrested at metaphase II; it was concluded that sperm that do not activate the oocyte might continue decondensing the chromatin. However, the oocyte prevents

male pronucleus formation before the female one, mostly by causing premature chromatin condensation in the sperm and by duplicating the sperm centrosome.

The functional role of the sperm tail in early human embryonic growth is not known. In microinjection experiments, it was demonstrated that injection of isolated sperm segments (heads or flagella) could permit oocyte activation and bipronuclear formation. However, a high rate of mosaicism was observed in the embryos with disrupted sperm, suggesting that the structural integrity of the intact fertilizing spermatozoon appears to contribute to normal human embryogenesis (143). In addition, oocytes injected with mechanically dissected spermatozoa, although capable of pronuclear formation, did not undergo normal mitotic division. The lack of a bipolar spindle, in combination with mosaicism, suggested abnormalities of the mitotic apparatus when sperm integrity is impaired after dissection (144).

Occasional occurrence of paternal inheritance of mtDNA has been suggested in mammals, including humans (145). While most such evidence has been widely disputed, of particular concern is the documented heteroplasmic or mixed mtDNA inheritance after ooplasmic transfusion (146). Indeed, there is evidence that heteroplasmy is a direct consequence of ooplasm transfer, a technique that was used to "rescue" oocytes from older women by injecting ooplasm from young oocytes. ICSI has inherent potential for delaying the degradation of sperm mitochondria. However, paternal mtDNA inheritance after ICSI has not been documented.

Putative Dysfunctions Resulting from Aberrant Delivery of mRNAs

New evidence has challenged the traditional view of the transcriptional dormancy of terminally differentiated spermatozoa. Several reports indicated the presence of mRNAs in ejaculated human spermatozoa (147, 148). It has been hypothesized that these templates could be critically involved in late spermiogenesis, including a function to equilibrate imbalances in spermatozoal phenotypes brought about by meiotic recombination and segregation, but also that they could be involved in early postfertilization events such as establishing imprints during the transition from maternal to embryonic genes. Others have instead proposed that mature spermatozoa are a repository of information regarding meiotic and postmeiotic gene expression in the human and are likely to contain transcripts for genes playing an essential role during spermiogenesis. The use of the whole ejaculate as a wholly noninvasive biopsy of the spermatid should therefore be evaluated (149).

A nonexhaustive list of transcripts include c-myc, HLA class 1, protamines 1 and 2, heat shock proteins 70 and 90, ß-integrins, transition protein-I, beta-actin, variants of phosphodiesterase, P receptor, and aromatase, as well as an extended pattern of several transcripts encoding factors (NF_kB, HOX2A, ICSBP, JNK2, HBEGF, RXRβ, and ErbB3), revealing a wide range of transcripts in mammalian, including human, sperm. The presence of residual DNA and RNA polymerase activity within the sperm chromatin has also been formerly reported (150–154).

Complementary investigations have indicated that in spite of a high degree of DNA packaging within the human sperm head, chromatin retains some features of active chromatin, mainly acetylated histones, and the arrangement of certain chromatin domains into the nucleosomes (155–158). The existence of translational activities in human sperm during capacitation and AR has been described, which could also explain the presence of mRNA in mature sperm (159).

It is possible that if the mRNAs accumulated in the sperm nucleus are not residual nonfunctional materials, they might be viewed as the male gametes' contribution to early embryogenesis (160). Delivering spermatozoa RNA to the oocyte has been demonstrated in mice (161) and humans (147). Some sperm transcripts encoding proteins known to participate in fertilization and embryonic development have been specifically detected in early embryos after IVF failure, while they have not been found in the oocyte. Thus, human spermatozoa could act not only as genome carriers but also as providers of specific transcripts necessary for zygote viability and development before activation of the embryonic genome.

In this regard, Avendaño et al. (162) recently examined the possibility that some mRNA transcripts present in mature ejaculated human spermatozoa can survive after fertilization and might therefore play a function in the new cell. Two transcripts (PSG1 and HLA-E) with known roles in implantation were studied; these transcripts are human specific (absent in hamster eggs and present in human sperm). Results demonstrated that these messengers were present 24 hours after fertilization in a heterologous model (human sperm injected into hamster oocytes by ICSI), indicating that the mature spermatozoa deliver mRNA into the oocyte and that these molecules can selectively survive and possibly play a role in embryo development.

Aberrant Embryogenesis Secondary to Nuclear/Chromatin Anomalies

In human spermatozoa, 15%–20% of histones are retained in the nucleus to coexist with protamines. Hypothetically, nucleohistone regions of sperm chromatin mark DNA sequences for distinctive processing during fertilization and early embryogenesis. Nazarov et al. (163) have proposed a novel model for the nuclear architecture of human spermatozoa. Elaborate nonrandom organization of human sperm chromosomes at different structural levels, starting from the DNA packing by protamines up to the higher-order chromosome configuration and nuclear positioning of chromosome territories, has been discovered. Zalensky and Zalenskaya (164) have put forward a hypothesis that the unique genome architecture in sperm provides a mechanism for orchestrated unpacking and ordered activation of the male genome during fertilization, thus offering an additional level of epigenetic information that will be deciphered in the descendant cells.

During the process of mammalian spermiogenesis, a significant reorganization of the chromatin structure occurs involving the sequential substitution of somatic histones with protamines. In the human sperm nucleus, approximately 15% of the basic nuclear protein complement is maintained as histones. Human testis/sperm-specific histone H2B (hTSH2B) is a variant of the histone H2B expressed exclusively in spermatogenic germline cells and present in some mature sperm cells. Thus, this protein marks a subpopulation of sperm cells in the ejaculate (165, 166). Using indirect immunofluorescence, Singleton et al. (165) examined the influence of hTSH2B on ZP binding and sperm head decondensation in amphibian egg cell-free extract. As suggested by previous studies, they reported that hTSH2B can be localized in only approximately 30% of sperm cells within a given ejaculate. The investigators established that the presence of hTSH2B does not influence sperm ZP binding capacity. Finally, it was found that decondensation occurred more rapidly and to a greater extent in those cells containing hTSH2B. It is proposed that the presence or absence of hTSH2B within spermatozoa

influences pronuclei formation and the activation of paternal genes after fertilization and during early embryonic development.

Esterhuizen et al. (167) evaluated the role of chromatin packaging (CMA3 staining), sperm morphology during sperm-zona binding, sperm decondensation, and the presence of polar bodies in oocytes that failed IVF. Odds ratio analyses indicated that being in the $\geq$60% CMA3 staining group resulted in a 15.6-fold increase in the risk of decondensation failure, relative to CMA3, with staining of <44%. Therefore, sperm chromatin packaging quality and sperm morphology assessments are useful clinical indicators of human fertilization failure.

Ejaculated human spermatozoa may present various degrees of DNA damage. Different theories have been proposed to explain its origin (125, 168–171): [1] damage could occur at the time of or be the result of DNA packing during the transition of histone to protamine complex during spermiogenesis; [2] DNA fragmentation could also be the consequence of direct oxidative damage that has been associated with antioxidant depletion, smoking, xenobiotics, heat exposure, leukocyte contamination of semen, and presence of ions in sperm culture media; and [3] DNA damage could be the consequence of apoptosis.

The presence of apoptosis in ejaculated spermatozoa could be the result of various types of injuries (125, 170, 171). 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

TABLE 2

Compelling evidence of the presence of somatic cell apoptotic markers in human ejaculated spermatozoa as reported by our laboratories.

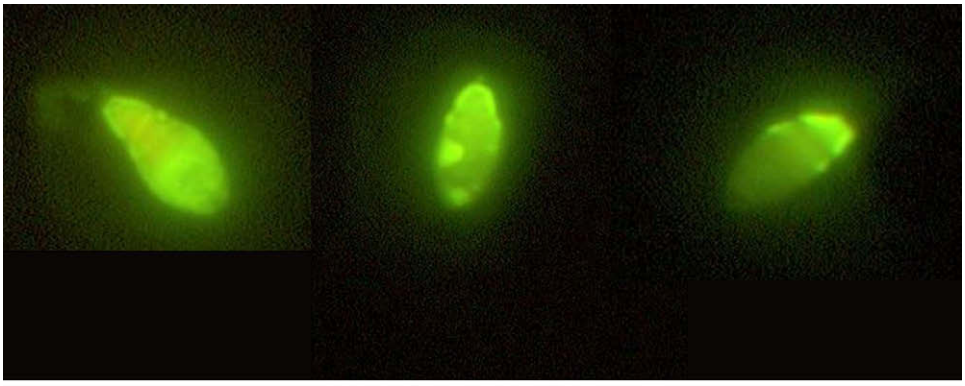
Authors	Evidence
Barroso et al. 2000 (125); Schuffner et al. 2002 (176); Weng et al. 2002 (177)	Early and late apoptotic markers were demonstrated using annexin V and TUNEL. Also, an important role of activated and nonactivated caspases was shown by using specific monoclonal antibodies.
Barroso et al. 2000 (125); Weng et al. 2002 (177)	The apoptotic markers: PS externalization and DNA fragmentation are expressed with a higher frequency in the fractions of sperm with low motility.
Weng et al. 2002 (177); Oehninger et al. 2003 (172)	Apoptotic markers are expressed with a significantly higher frequency in spermatozoa from infertile men when compared with fertile controls.
Weng et al. 2002 (177)	Caspase-3, the major executioner caspase, was demonstrated in human sperm in both active and inactive forms. Apparently, active caspase-3 was exclusively detected in the midpiece, where mitochondria and residual cytoplasm are present.
Taylor et al. 2004 (178)	Human sperm exhibit other members of the caspase family, caspase-7 and -9. By immunoblotting, we demonstrated the presence of inactive caspase-7 and caspase-9 in many samples, as well as active caspase-7 and caspase-9 in samples of infertile men.
Taylor et al. 2004 (178)	Human sperm possess the AIF. By immunoblotting, we have demonstrated that human sperm express AIF. Additionally, sperm expresses unique poly-ADP-ribose polymerase, a specific caspase substrate found in somatic cells.
Taylor et al. 2004 (178)	Caspase activation can be triggered in ejaculated human sperm by the mitochondrial disruptor staurosporine, which significantly enhanced caspase activation and DNA fragmentation.
Taylor et al. 2004 (178); Castro et al. 2004 (179)	Human sperm did not trigger or exhibit any response to Fas ligand in experiments testing caspase activation, PS translocation, or DNA fragmentation.
Duru et al. 2001 (180); Oehninger et al. 1995 (181)	Hydrogen peroxide (H_2O_2), the most damaging ROS in sperm, induces the expression of apoptotic markers by increasing PS translocation and DNA fragmentation.
Barroso et al. 2000 (125)	Ejaculated human sperm show a strong correlation between ROS production and DNA fragmentation, linking mitochondrial dysfunction and expression of apoptosis markers.
Barroso et al. 2006 (128)	Ejaculated sperm show a strong correlation between disruption of the mitochondrial potential membrane and PS translocation.

Note: AIF = apoptotic inducing-factor (AIF); PS = phosphatidylserine; TUNEL = TdT-mediated dUTP Nick-End Labeling; DNA = Deoxyribonucleic acid; ROS = Reactive oxygen species.

Barroso. Paternal contributions to the human embryo. Fertil Steril 2009.

FIGURE 4

Three different patterns of DNA fragmentation (TUNEL assay) frequently observed in infertile men (left: granular appearance throughout the sperm head; center: predominance of vacuolated appearance; right: homogeneous fluorescence throughout nuclear area).



Note: TUNEL = TdT-mediated dUTP Nick-End Labeling.

Barroso. Paternal contributions to the human embryo. Fertil Steril 2009.

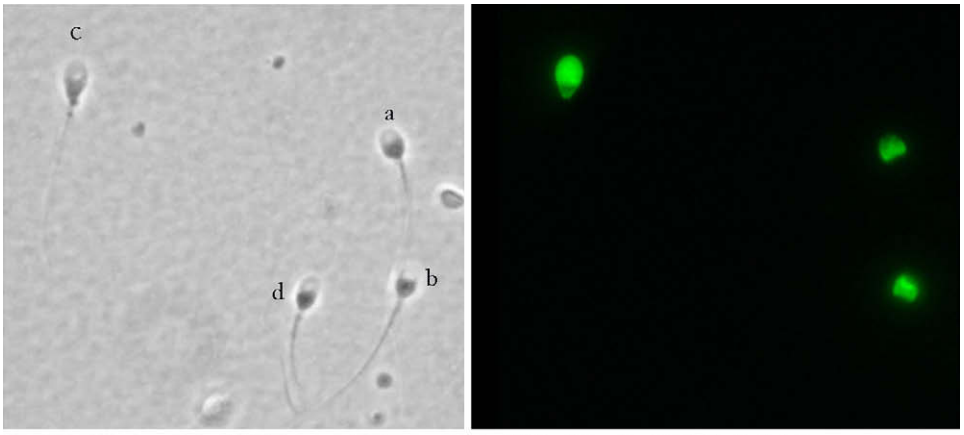
ROS and other mediators of inflammation/infection), or seminal (ROS, lack of antioxidants, or other causes) levels. In addition, apoptosis could be triggered by factors present in the female tract. In vitro, apoptosis could be triggered upon incubation with inappropriate culture media or other manipulation procedures. Irrespective of the stimulus, spermatozoa undergoing apoptosis and unrecognized by currently used methodologies may be dysfunctional. More dramatically, they may pose the risk of carrying a damaged genome into the egg, resulting in poor embryo development, miscarriage, or birth defects (170).

Compelling evidence indicates the presence of somatic cell apoptosis markers in human ejaculated spermatozoa (168–172). Evidence gathered in our laboratories is summarized in Table 2 (125, 128, 173–177). The oocyte has the capability to repair the DNA damage as oocytes fertilized by DNA-damaged spermatozoa did not develop further in vitro when they were cultured in the presence of inhibitors to DNA repair (178). But the capacity of the oocyte to repair is limited and is related to the degree of sperm DNA damage. The fertilization capacity of apoptotic sperm has been observed at the same rate as intact spermatozoa; however, the in vitro embryo development to the blastocyst stage is closely related to the integrity of the DNA (179).

It has been known for many years that the chromatin of the mature sperm nucleus can be abnormally packaged (180). In addition,

FIGURE 5

Morphologically normal sperm with fragmented DNA (normal-SFD) in the same semen sample of patients currently undergoing ICSI. Simultaneous assessment of normal sperm morphology (phase contrast, unstained) and DNA fragmentation (TUNEL) after swim-up separation (left side: phase contrast; right side: TUNEL fluorescence). (A) Normal spermatozoon with DNA fragmentation, (B) abnormal spermatozoon (slightly abnormal form) with DNA fragmentation, (C) morphologically abnormal spermatozoa (severely amorphous) with DNA fragmentation, and (D) morphologically abnormal spermatozoa (tapered form) without DNA fragmentation.



Note: DNA = Deoxyribonucleic acid; ICSI = Intracytoplasmic sperm injection; TUNEL = TdT-mediated dUTP Nick-End Labeling.

Barroso. Paternal contributions to the human embryo. Fertil Steril 2009.

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 of men and poor semen parameters (170–172, 181). Endogenous nicks in DNA are normally expressed at specific stages of spermiogenesis in different animal models; these endogenous nicks are evident during spermiogenesis but are not observed once chromatin packaging is completed. It is postulated that an endogenous nuclease, topoisomerase 11, creates and ligates nicks to provide relief of torsional stress and to aid chromatin rearrangement during protamination. (182–186).

Several studies have shown that sperm DNA quality had robust power to predict fertilization in vitro (187–189). Tomlinson et al. (190) reported that the only parameter showing a significant difference between pregnant and nonpregnant groups in IVF was the percentages of DNA fragmentation assessed by in situ nick translation. The sperm chromatin structure assay (SCSA) has been proposed as a diagnostic tool to predict fertilization by evaluating sperm DNA stability (191). The SCSA measures susceptibility to DNA denaturation in situ in sperm exposed to acid for 30 seconds followed by acridine orange staining. The use of flow cytometry in the SCSA increases its dependability.

Duran et al. (123, 192), studied a large infertility population undergoing IUI in a prospective cohort fashion. A total of 119 patients underwent 154 cycles of IUI. DNA fragmentation evaluated by terminal deoxynuceotidyl transferase-mediated UTD nick-end labeling (TUNEL) and acridin orange staining was measured. The investigators reported that sperm DNA quality played a major role as a predictor of pregnancy under such in vivo conditions. Figure 4 shows three different patterns of DNA fragmentation frequently observed in infertile men. More studies are needed to determine the clinical significance of these finding in terms of fertilization and developmental potential.

Avendaño et al. (193) recently reported that infertile men can present DNA fragmentation in the morphologically normal spermatozoa recovered post-swim-up. In addition, using simultaneous evaluation of DNA integrity and sperm morphology in the same sperm cell (Figure 5), the same investigators evaluated morphologically normal sperm with fragmented DNA (normal-SFD) in a fraction of the same semen sample of patients currently undergoing ICSI. Findings demonstrated a significant and negative correlation between the percentage of normal-SFD and embryo quality and, more importantly, with pregannсy outcome (194).

Muratori et al. (195) identified a group of round bodies without a nucleus of different size and density in human semen. These bodies are positive for the fluorochrome merocyanine 540 staining after which they were named. It has been postulated that these elements are residues of apoptotic germ cells, demonstrated by the presence of some apoptotic markers (caspase activity, FAS, p53 and Bcl-x, and DNA fragmentation). Besides, it was also observed that these elements are especially frequent in oligoasthenoteratozoospermic patients. An apparently logical explanation that the investigators have developed is that these findings suggest that these apoptotic bodies have somehow escaped from testicular or epididymal phagocytosis.

Using flow cytometry, it is possible to distinguish sperm from M540 bodies by labeling samples with nuclear probes because the latter fail to stain M540 bodies. In two studies performed with flow cytometry to study sperm ubiquitination and DNA fragmentation in which M540 bodies were both included and excluded, it was demonstrated that M540 bodies largely affected the results (196). Future investigations should take into consideration the information reported by these studies to thoroughly analyze the degree of DNA fragmentation in sperm subpupulations (197).

CONCLUSIONS

This review highlighted the concept that the spermatozoon has a very dynamic and critical participation in normal embryogenesis that clearly extends beyond the fertilization process. Progress is slowly being made related to the unveiling of molecules involved in sperm-oocyte interaction and regulators of fertilization and post-fertilization developmental steps. Furthermore, we presented unequivocal clinical evidence that defective spermatozoa that penetrate the oocyte may cause arrest of development at multiple levels during embryo preimplantational development. Additional data suggest that sublethal effects can be "carried over" after implantation, resulting in untoward embryonic/fetal defects. These findings highlight the need for continuous monitoring of ART results.

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