Mini Review

Use of Fluorescent Dyes for Readily Recognizing Sperm Damage

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Abstract

Sperm is produced by the testis and mature in the epididymis. For having a successful conception, the fertilizing sperm should have functional competent membranes, intact acrosome, functional mitochondria and an intact haploid genome. The effects of genetic and environmental factors result in sperm vulnerability to damage in the process of spermatogenesis and maturation. In recent years, the feasibility of detecting sperm damage is enhanced through the advances in technologies like fluoscerent staining techniques assisted with fluorescence microscope, flow cytometry and computer analysis systems. Fluoscerent staining techniques involve the use of fluorescent dyes, either directly or indirectly for binding them with some ingredients of sperm and evaluating the damage of the structure or function of the sperm, *i.e.* membrane, acrosome, mitochondria, chromosome or DNA.

Keywords: Acrosome, Chromatin/DNA, Fluorescent staining, Motichondrial, Motility, Sperm damage.
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Introduction

A large number of spermatogenic cells undergo degeneration and apoptosis during the stages of spermatogenesis in mammals with normal fertility (1, 2). It is a physiological way for the renewal of spermatogenic cells and the organisms to clear the redundant or abnormal cells (1, 2). Under pathological conditions, genetic factors, environmental factors or the combination of both genetic and environmental factors would result in sperm vulnerability to damage during the process of spermatogenesis and maturation. The common causes that lead to sperm damage are orchitis, epididymitis, varicocele, cryptorchidism, high temperature, hormone level variation, medication, radiotherapy and chemotherapy, and environmental pollution.

In addition, the application of assisted reproductive techniques (ART) such as artificial insemination (AI), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) may damage the sperm. When using ART, the sperm is not directly injected inside the female genital tract at ejaculation. Instead, the sperm is first collected in a bottle or tube and it undergoes some pretreatment, screening or cryopreserved, which might result in some damage to the sperm (3).

Research on sperm damage has always been one of the hotspots in the field of reproductive medicine. In the past, only the ordinary optical microscope could be used to observe semen and assess the conventional parameters of semen, *e.g.* sperm density, vitality, motility and morphology. However, with the development of technology, fluorescent staining techniques are more widely applied to evaluate sperm characteristics including the sperm membrane integrity, acrosome status, mitochondria activity, chromatin and DNA integrity. Nowadays, we can analyze the type and extent of sperm damage more accurately through the application of fluorescent microscope, flow cytometry and computer analysis system. In this acticle, we discuss about the application of fluorescent staining technique in detecting sperm damage.

Detection of Sperm Motility: Sperm motility refers to the percentage of the progessive sperm (fast progressive and slow progressive sperm) in semen (4). The progressive sperm are able to swim up towards the egg and fertilize it. According to the World Health Organisation (WHO) manual criteria (2010), spermatozoa are categorized as progressively motile, non-progressively motile and immotile by computer-assisted sperm assay (CASA) in the semen routine examination (5). Although it is very convenient to detect the sperm motility by CASA, few reports detecting sperm motility by fluorescent staining techniques are available.

However, Maria E. Cabrillana et al. used a fluorescent thiol-selective labeling agent, monobromobimane (mBBr) which is specifically attached to outer dense fibers 1 (ODF1) to label the sperm for studying the effect of N-terminal domain of ODF1 on sperm motility. They found out that ODF1 was highly responsible for mBBr fluorescence detection in the sperm tail and ODF1 N terminal domain were related to sperm motility (6). Vazquez JM et al. reported that staining using fluorescence dye, Hoechst 33342, didn't affect the motility of spermatozoa (7).

Detection of Sperm Viability: The plasma membrane surrounds the entire sperm cell holding together its organelles and intracellular components and by its semi-permeable features maintains the chemical gradient of ions and other soluble components (3). If the sperm plasma membrane is not functionally intact, the sperm is considered deteriorated (dead) and *in vivo* cannot fertilize the sperm (3).

Using one fluorescent dye can detect the sperm viability. These fluorescent dyes can be grouped into two categories. One group is the membrane-impermeable dyes which are able to pass through the damaged plasma membrane of dead sperm and be observed directly under a fluorescence microscope, *e.g.* EB, EH, PI, YoPro-1, ToPro-3, TOTO and Hoechst 3358. Another group is acylated membrane dyes that have no fluorescence, but can turn into fluorescent substances when passing the intact plasma membrane and enter the living sperm, *e.g.* CFDA, CAM, SYTO-1 and SYBR-14. However, researchers prefer to use double staining with two fluorescent dyes to achieve more accurate results, *e.g.* SYBR-14/PI, YO-PRO-1/PI, AnnexinV-FITC/PI and AnnexinV-PE/7-ADD. Hence, they group the sperm into living sperm, apoptotic sperm and dead sperm.

Detecting the lipid peroxidation can also reflect the membrane's function. The conventional techniques usually detect the end-product of lipid peroxidation, while the fluorescent staining techniques can directly detect endogenous phospholipid and cholesterol with fluorescent probe (8). For example, using a fluorescent lipid peroxidation reporter molecule C_{11}BODIPY (581/591), which shifts its fluorescence from red to green when challenged with oxidizing agents, can tract, quantify and locate the lipid peroxidation (8, 9).

Detection of Plasma Membrane Fluidity: The plasma membrane fluidity is the basic requirement for the sperm's motor ability and structure integrity. Changes in membrane fluidity could theoretically impede the assembly and activation of signal transduction pathways that are critical to the fertilization process (10).

Aboagla E.M. and Terada T. used merocyanine 540/Yo-Pro-1 staining to study the influence of trehalose on membrane fluidity and to group the sperm into three subpopulations (11). Cells that were stained with Yo-Pro-1 were classified as dead sperm; cells unstained with Yo-Pro-1 showing a low merocyanine 540 fluorescence signal were the sperm with low membrane fluidity; and cells with more than the minimal merocyanine 540 signal were the sperm with high membrane fluidity (11). Julie Baumber and Stuart A. Meyers used lipophilic fluorescent dye, merocyanine 540, to stain the sperm cell membrane, and found out that the lipid order in membrane decreased during the process of sperm capacitation (12).

Detection of Acrosome Status: The acrosome integrity of sperm is a key for successful fertilization. The non-fluorescent staining methods have long been used to detect the sperm acrosome status, *e.g.* eosin/nigrosin staining, Giemsa staining, Papanicolaou staining and brilliant blue staining. Currently, the fluorescent staining technique is more widely used to detect acrosome status. The most commonly used fluorescein isothiocyanate (FITC)-labeled lectins are FITC-PSA (13, 14), FITC-PNA (15), FITC-ConA and FITC-RCA-II.

Alvarez M. et al. used FITC-PNA/PI staining to evaluate the effect of sperm concentration at freezing on post-thaw semen quality (16). FITC-PNA negative/PI positive were nonviable sperm with intact acrosome; FITC-PNA positive/PI neg-

ative were viable sperm with damaged acrosome; and FITC-PNA negative/PI negative were viable sperm with intact acrosome (16). Besides, chlortetracycline (CTC) (14), fluorescein-labeled mannosylated Bovine Serum Albumin (BSA), fluorescein-labeled acrosome-specific monoclonal antibody and eosinophilic probe Lysotracker Green TM can also be used to detect the acrosome status.

Detection of Mitochondria Activity: The mitochondria activity is a key indicator of sperm function. Mitochondria, localized in the mid-piece area of sperm flagellum, produce ATP to support sperm motility. The commonly used fluorescent dyes to detect mitochondria activity include Rhodamine 123 (R123), Mitotracker Green TM (17), Mitotracker Red CMXROs, Mitotracker Deep Red 633 (M-22426) (18), Mitotracker Orange TM (19), $DiOD_6(3)$ and JC-1 (20).

Kimberly A. Terrell et al. used the MitoTracker Red CMXROs labeling the mitochondria to study the cryopreservation-induced damage to spermatozoa. The spermatozoa were classified as five morpho-types: structurally-normal, midpiece droplet, flagellar droplet, spermatid and others (including head deformities, malformation of the midpiece or flagellum) (21). Leyland Fraser et al. used R123/PI double staining to evaluate the function of mitochondria. Sperm cells displaying only green fluorescence at the mid-piece region were the viable sperm with functional mitochondria (22). Branko Zom et al. used $DiOD_6(3)$/PI double staining to measure the mitochondria membrane potential (MMP) of sperm. The cells with high fluorescence signal were the sperm with normal mitochondria membrane potential, while the cells with low fluorescence signal were apoptotic cells (23).

Detection of Chromatin and DNA Damage: The common methods to detect sperm chromatin and DNA damage aresperm chromatin structure assay (SCSA), sperm chromatin dispersion (SCD) test, Comet Assay, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) and fluorescence in situ hybridization (FISH).

SCSA test (also known as sperm DNA fragmentation test or sperm chromatin fragmentation test) is original and gold standard for sperm DNA fragmentation screening which delivers a highly accurate measure of male reproductive health (24). Acridine orange (AO) binding with the double-stranded DNA as monomeric form can show green fluorescence, while binding with single-stranded DNA as polymer can show red or yellow fluoscence. SCSA use the AO and flow cytometry to get the parameters including DFI and HDS, and to evaluate the sperm chromatin integrity and fertilizing capacity. Helena Oliveira et al. detected the effect of lead chloride on sperm chromatin integrity by SCSA (25) and if the exposure time of lead chloride to mice weretoo short, they could not reach the same positive results (25).

SCD is a test that spreads the sperm suspension mixed with agarose flat out on slides, dissolves cell and digests nucleoprotein with acid treatment. Later it is stained with DAPI or Diff-Quik reagent, and finally observed by fluorescence or light microscope (26, 27). Diffusion halo can be observed in the normal sperm, while no halo or a very small halo can be observed in the damaged sperm.

Comet assay, also known as single cell gel electrophoresis (SCGE), is based on a principle that when the sperm DNA breaks, DNA supercoils become loose and the negative charges are exposed. Under the electric field force, DNA fragments move out from the nucleus and migrate to the positive electrode in sperm with fragmented DNA. Then, they are stained with EB, DAPI or other dyes, and are observed under fluorescence microscope. The damaged sperm shows a comet-like tail. According to the fluorescence intensity and tail length, we can evaluate the extent of sperm DNA fragmentation. J. Ribas-Maynou et al. used alkaline and neutral Comet assay to detect the sperm damage of different groups of patients including athenoteratozoospermic (AT) with or without varicocele, oligoasthenoteratozoospermic (OAT) or balanced chromosome rearrangements. They found out that OAT, AT and AT with varicocele presented high percentages of ssDNA and dsDNA fragmentation (28).

TUNEL is based on a principle that endogenous nucleic acid enzymes of apoptotic sperm are activated so that the DNA is cut into a notch or fragments of 180-200 *bp* having 3'-OH ends. TDT is used to transfer the dUTP labeled with fluorescent marker (*e.g.* Texas Red (29) and FITC (25)) to terminal 3'-OH, and then they can be observed by flow cytometry or fluorescence microscopy.

FISH uses fluorescent labeled probe to hybridize them to metaphase chromosome or interphase chromosome for detecting the abnormalities of sperm chromatin and at the same time analyzing the aneuploidy rate of chromosomes. Different

probes have different colors, and several probes can be applied to the same sample. With the techniques of FISH and specific probe to some chromosomes, we gained the rates of becoming amphiploid of chromosomes 1, 2, 4, 6, 9, 12, 13, 15, 16, 18, 20, 21, 22, X and Y. Katerina Chatzimeletiou et al. uesd FISH to detect chromosomal abnormalities of the sperm from a man whose wife had a partial mole, including chromosomes 13, 15, 16, 18, 21, 22, X, and Y, and found out that the semen sample analyzed had a high incidence of abnormal morphology and increased diploidy (29).

Also, we can use chromomycin A3 (CMA3), toluidine blue (TB) or aniline blue (AB) to detect the sperm chromatin integrity/maturity (30, 31). If sperm chromatin is well differentiated, it is in the form of protamine-DNA complex and will not be stained by CMA3, TB or AB; if the protamine of chromatin is defective, the sperm nucleus would be stained. Mohammad Bozlur Rahman (2011) used CMA3 staining and found out that heat stress leading to sperm damage mainly causes protamine defects (32). Furthermore, CMA3 is the most sensitive and specific test for sperm nuclear maturity (33). Besides, the fluorescent staining techniques are also applied for real-time fluorescence quantitative PCR (34) and gene chip (35) technology by using the probe with fluorescent marker to detect the target gene.

Conclusion

With the development of various technologies, more and more sperm minor structural damages are found; the mechanisms of damage to sperm by various external factors such as heavy metals, cryopreservation, drugs and drinks are better recognized than before. Sperm chromatin, DNA, multiple gene or single gene abnormalities are among the research hotspots of reproductive medicine. Detection of sperm damage not only assesses the male fertility, infertility and subclinical infertility, but also detects the integrity of sperm that are used in assisted reproductive technology. Fluorescent staining technique can detect the target substances qualitatively, quantitatively and positionally, so it is considered as an important means for detection of sperm damage. However, there are still some problems for the application of fluorescent staining in clinical settings. The typical problems are laborious staining technique, high cost of reagents, standardization, and verification of the fluorescent dyes security for assisted reproduction In spite of the aforementioned problems, fluorescent staining technique, as a new technology, will become more important in the future and will be widely used in the study of the mechanisms of various sperm damage.

Conflict of Interest

The authors declare no conflict of interest.

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