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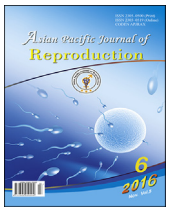

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Mitochondrion: Features, functions and comparative analysis of specific probes in detecting sperm cell damages

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ABSTRACT

In assisted reproduction, ejaculated semen is either washed immediately for in vitro fertilization or diluted and processed further for optimal low temperature liquid- or cryo-preservation. The extended handling of semen makes sperm vulnerable to organelle and/or membrane damages. In addition to several important functions, mitochondrion is chiefly responsible for production of detrimental free radicals in spermatozoa. Therefore, direct evaluation of mitochondrial activitymight be useful in semen evaluation protocol as an alternate, more objective and desirable parameter of spermatozoa quality. This is further aided by availability of wide spectral range of fluorescent probes that has advantage of simultaneous multi-parametric assay. Even with availability of several mitochondrial-specific probes, it is sometimes difficult for an investigator to select a most suited dye for experimentation. Therefore, in this review the literature pertaining to role of the mitochondria in the sperm cell, focusing on morphology, maintenance of trans-membrane potential, mechanism involved in generation of reactive species, and comparative analysis of a number of probes used for evaluating mitochondrial function in sperm cells are discussed. Our intention is to present concise information on the technical aspects of various probing methods, and this might be useful for investigators to design experimental approach by proper selection of the dye and for accurate interpretation of the results.

1. Introduction

The mammalian fertilization is a very complex process requiring a well-orchestrated series of events played by oocyte and sperm cell. For successful fertilization, one of the most essential attributes of spermatozoa is motility, required for transport through female genital tract, as well as in the approach to the waiting oocyte, and finally as an aid in penetration of zona pellucida [1]. Sperm motility is the result of flagellar movement of the sperm tail, achieved by ATP-derived energy, produced in mid-piece located mitochondrion. Evaluation of motility in assisted reproduction and in semen freezing laboratories is considered as an important attribute of sperm viability and in-direct measure of metabolism [2,3]. Therefore, it flows naturally to surmise that measurements of mitochondrial function might be useful as an alternate, more objective measure of sperm quality. It is crucial to understand the complexity of the mitochondrial compartments related to its functionality and to evaluate specific fluorescent probes used in assessing the morpho-functional features of mitochondria in different living- or fixed-cell types. Given the pivotal role mitochondria play in cellular life, this review covers mitochondrial features and functions vital in interpreting results and at the same time, elucidating finer points of various fluorescent probes employed to evaluate sperm mitochondrion.

2. Brief background of mitochondrion science

The earliest records on intracellular structures that probably represent mitochondria go back to the 1840s [4]. However, Altmann [5] was the first to recognize the ubiquitous occurrence of these structures, calling them “bioblasts” and concluding that they were “elementary organisms” living inside cells and carrying out vital functions. Benda introduced the name ‘mitochondrion’ in 1898 [6]. The word originates from the Greek “mitos” (thread) and “chondros” (granule), referring to the appearance of these structures during spermatogenesis. A brief history of literature related to development of knowledge source related to mitochondrion has been provided in Table 1.

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Table 1
Chronology of advances in mitochondrion science.

Time	Advance	Authors
2002	Mito SOX	Batandier *et al.* [7]
1996	MitoTracker®	Poot *et al.* [8]
1991	JC-1/JC-9	Smiley *et al.* [9]
1988	TMRM/TMRE	Ehrenberg *et al.* [10]
1981	Carbocyanines	Johnson *et al.* [11]
1980	Rhodamine 123	Johnson *et al.* [12]
1975	Evidence for electron transport-linked proton pumps	Papa *et al.* [13]
1967	Separation and characterization of inner and outer membrane	Chappell *et al.* [14]
1963	Mitochondria contains DNA	Nass *et al.* [15]
1963	Energy-linked uptake of Ca^{2+}	Chappell *et al.* [16]
1961	Chemiosmotic hypothesis	Mitchell [17]
1958	Protein synthesis by mitochondria	McLean *et al.* [18]
1957	Termed 'Powerhouse of the cell'	Siekevitz [19]
1952	Electron micrograph of mitochondria	Palade [20]
1948	Isolation of intact mitochondria	Hogeboom *et al.* [21]
1934	Isolation of mitochondria by cell fractionation	Bensley *et al.* [22]
1925	Association of CytC with cellular structures	Keilin [23]
1900	Janus Green B	Michaelis [24]
1898	Term 'mitochondrion' was coined	Benda [6]
1890	Mitochondria as 'Bioblast'	Altmann [5]

TMRE, tetramethylrhodamine ethyl ester; TMRM, tetramethylrhodamine methyl ester.

3. The sperm mitochondria

The mitochondria of mammalian spermatozoa are restricted to the mid-piece of the flagellum. They wrap helically around the outer dense fiber axoneme complex in a species-specific manner during spermiogenesis to form a cylinder-shaped mitochondrial sheath [25]. Within the sheath, adjacent mitochondria associate both end to end and along their lateral surfaces. This positioning of a concentrated array of mitochondria adjacent to the flagellum is believed to be an efficient way to provide at least some of the energy required for motility [26]. The typical mammalian sperm mid-piece contains approximately 50–75 mitochondria with one copy of mitochondrial DNA in each [27]. The fluorescent probes stain mid-piece of the spermatozoa containing mitochondria for functionality evaluation.

3.1. Features

The mitochondrion (0.75–3 μm in dia, [28]) is a double membrane-bound organelle found in all eukaryotic organisms (except red blood cells) where they make up as much as 10% of the cell volume [29]. They are pleomorphic organelles with structural variations depending on cell type, cell cycle stage and intracellular metabolic state [30–32].

Morphologically, mitochondrion has two distinct membranes, the outer mitochondrial membrane (OMM) with smooth boundaries and the inner mitochondrial membrane (IMM) forming many invaginations and tubes covering almost whole of the lumen, called cristae. The inner membrane and the lamellar structures are connected by narrow tube-like connections, called the cristae junctions [33]. It is remarkable to note that the inner and the outer membrane have completely different protein content and are functionally distinct [30,34]. Porin (30–35 kDa) is the most abundant protein of the OMM. Through the pores of the trans-membrane channel located in porins, passage of ions and small molecules is facilitated [35,36]. The maximal molecular weight of uncharged molecules, which can pass through the porins, is about 5 kDa [30,37]. For transport of proteins <5 kDa, binding of signaling sequence at their N-terminus with a large multi-subunit protein called 'translocase' of the OMM is required which then actively moves them across the membrane [38]. Mitochondrial pro-proteins are imported through specialized translocation complexes. Any damage to outer membrane permits proteins in the inter-membrane space to leak into the cytosol, leading to eventual cell death [39]. Outer membrane also contains many enzymes, which are involved in such diverse activities as degradation of tryptophan and oxidation of epinephrine.

The inner membrane contains 76% protein, which is more than any other cellular membrane [34]. It is a tight permeability barrier, with functional consequences. The IMM encloses chemiosmotic apparatus for energy production [40,41]. In the process of oxidation (ATP production) of glucose and free fatty acids by enzymes in the mitochondrial respiratory chain, protons (H^+) are pumped into the cell cytosol. The resulting proton gradient results in development of electrostatic potential across the inner membrane [42]. Cation fluorescent probes in differentiating between functional or apoptotic mitochondrion exploit this property of the inner membrane (Figure 1). Mitochondrial membrane potential (MMP, $\Delta\Psi m$) is an important index of the bio-energetics state of the spermatozoa. Due to folding of the inner membrane (cristae), it has much larger surface area in comparison to outer membrane. The adenosine-5'- triphosphate (ATP) synthesis complexes are located in the cristae. The number and size of cristae depends upon energy demand of that particular cell [34]. In addition to being home to 151 types of proteins, inner membrane is rich in an unusual phospholipid, 'cardiolipin' which makes it impermeable [30,38,43]. Binding property of fluorescent probes such as 10-*N*-nonyl acridine orange and Mito-ID Red with cardiolipin is exploited to measure functional status of sperm mitochondria (Table 2).

The matrix is the space enclosed by the IMM containing about 2/3rd of the total proteins in a mitochondrion, hundreds of enzymes, special ribosomes, tRNA and several copies of mitochondrial DNA [30,44]. The mitochondria-associated ER membrane is another structural element that is increasingly recognized for its critical role in cellular physiology and homeostasis [45,46].

3.2. Functions

Mitochondria are cellular organelles that play a key role in maintaining the cellular bio-energetic- and ion-homeostasis and are producers of free radicals [47] in spermatozoa. At the same time, it also plays a central role in regulation of apoptosis (programmed cell death). Similar to 'Janus', the mitochondrion presents two faces looking both forward and backward. On the one hand it is involved in the maintenance of viability and vitality, and on the other side plays a central role in the regulation of programmed cell death [48,49]. Thus, the mitochondrion may be considered the guardian of the gate between life and death [47]. Figure 2 depicts vital functions performed by mitochondrion.

3.3. ATP production

The principle role of a mitochondrion is production of ATP for energy homeostasis. The ATP production is achieved by

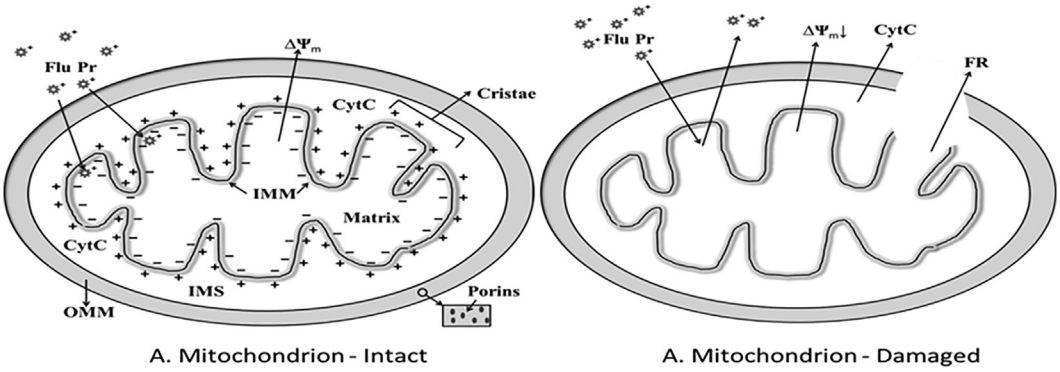

Figure 1. Functional features of sperm mitochondria.

Development of proton gradient to couple the electron transport to oxygen to form water in mitochondria results in production of ATP by the ATP synthase. This development of mitochondrial membrane potential ($\Delta\Psi m$) of the IMM can be sensed by the potentiometric probes (fluorescent probes, Flu Pr) like JC-1 that diffuse over the plasma membrane and OMM and accumulate in the IMM. While some of the potentiometric probes only fluoresce when intercalated in the IMM, others fluoresce after interacting with radicals generated by mitochondrion that convert them to fluorescence. Because of their negative membrane potential, mitochondria accumulate lipophilic cations. Once mitochondrion loses its integrity, the membrane proton gradient is lost and cytochrome C (CytC) required for electron transport chain diffuses out. With the outflow of CytC from IMS and free radicals from mitochondria, respiratory activity of mitochondrion ceases and potentiometric probes does not recognize IMM and no fluorescence is generated.

oxidation (aerobic respiration) of major products of glucose: pyruvate and NADH produced in the cytosol [31,51]. The production of ATP from glucose has an approximately 13-times higher yield during aerobic respiration compared to fermentation [59]. The transport of ATP and ADP across inner- and outer-membrane occurs with the help of specific proteins viz. ATP-ADP translocase and porin, respectively.

In the mitochondria, NADH and $FADH_2$ are produced in the matrix and cytoplasm via citric acid cycle and glycolysis, respectively. The energy from these molecules is transferred to O_2 in several steps via the electron transport system [31]. In the inner membrane, protein complexes (cytochrome C reductase, cytochrome C oxidase, NADH dehydrogenase or ubiquinone) perform the transfer. This incremental release of energy is then used to pump protons (H^+) into the inter-membrane space. The increase in the proton concentration in the inner membrane is responsible for development of strong electrochemical gradient ($\Delta\Psi m$). The potential energy of proton is utilized to synthesize ATP and inorganic phosphate. The proton can return to the matrix through the ATP synthase complex. This process is called chemiosmosis [60,61]. This process is efficient; however, a small percentage of electrons may prematurely reduce O_2 forming reactive oxygen species such as superoxide. This not only causes oxidative stress and therefore decline in the mitochondrial function but also contribute to aging [62].

3.4. Heat production

Under certain conditions, protons can re-enter the mitochondrial matrix without contributing to ATP synthesis, a process known as *proton leak or mitochondrial uncoupling*. This occurs due to the facilitated diffusion of protons by a proton channel called 'thermogenin' into the matrix [63]. The process results in the release of unharnessed potential energy of the proton electrochemical gradient as heat [31].

3.5. Ion homeostasis and storage of calcium ions

Mitochondria play a vital role in maintaining ion homeostasis in sperm as well as other cells (reviewed by [64]). Mitochondria can store calcium transiently, a contributing process for the cell's homeostasis of calcium (Ca^{2+}) and thus act as 'cytosolic buffers' [55,65]. Although the endoplasmic reticulum is the most significant storage site of Ca^{2+}; endoplasmic reticulum maintains a significant interplay with mitochondria for calcium storage [66]. Mitochondrial matrix calcium can reach the tens of micromolar levels, which is necessary for the activation of isocitrate dehydrogenase, one of the key regulatory enzymes of the Kreb's cycle [67].

3.6. Generation of free radicals

Reactive species (*e.g.* reactive oxygen species, superoxide anion, hydroxyl radical, hydroperoxyl radical, and reactive nitrogen species, nitric oxide, nitrogen dioxide, nitronium cation) are continuously generated in normal cellular processes and are essential to life [68]. However, when homeostasis of reactive species is disturbed (overproduced or when antioxidants are depleted), these free radicals cause oxidative stress, leading to irreversible cellular damages. As the main producers of free radicals in most cells, mitochondria play a key role in several pathologies involving oxidative stress (*e.g.*, aging and apoptosis and neurodegenerative diseases). ROS can be produced in the mitochondrial electron transport chain by electron reduction of molecular oxygen by electron donors such as flavor-proteins and semi-ubiquinone species. Hydrogen peroxide is the major radical species measured in isolated mitochondria [47]. Evaluating free radicals in the sperm cells provides valuable information regarding quality of semen samples.

3.7. Biogenesis of steroids in mitochondria

Mitochondria are essential sites for steroid hormone biosynthesis. Mitochondria in the steroidogenic cells of the adrenal, gonad, placenta and brain contain the cholesterol side-chain cleavage enzyme, P450scc, and its two electron-transfer partners, ferredoxin reductase and ferredoxin. This enzyme system converts cholesterol to pregnenolone and determines net steroidogenic capacity, so that it serves as the chronic regulator of steroidogenesis [69]. Mitochondria play important roles in biosynthesis of sex steroid hormones, and these hormones can also regulate mitochondrial function [70,71].

Table 2
Specific probes for measuring mitochondrial functionality.

Probes/Excitation/Emission spectra ($ë_{ex}/ë_{em}$)	Features/fluorescence	Relative merits/applications
MMP sensitive probes		
R123, 507/529	Cell-permeant, rapid uptake and equilibration, NF, Green	Apoptosis, MMP measurement, drug resistance [90], Highly specific for living cells [87], Quenching phenomenon when used in high concentration [91]
JC-1, 514/529 or 590 and JC-9, 522/535 or 635	Polychromatic fluorescence-green in monomeric and red in multimeric state, NF,	MMP measurement in heterogenous cell population [92] More consistent in response to depolarization than R123/$DiOD_6$(3) [84], More specific for mitochondria vs plasma membrane potential and Can be used to stain intact tissue or isolated mitochondria [93].
TMRE, 549/574 and TMRM, 548/573	Lipophilic, does not stain other organelles, NF, Orange	Reversible staining, quantitative measurement of MMP [94],Non-toxic, do not form aggregates, do not show binding dependent fluorescence [10], No inhibition of mitochondrial respiration by TMRM, intermediate by TMRE [47].
MitoTracker Red CMXRos and MitoTracker Red CM-H_2XRos, 578/599	Thiol-reactive, cell permeant, F, Red	For evaluation of oxidation status of sperm cells, Requires more sophisticated equipment [95].
RedoxSensor Red CC-1, 540/600	Enters cells passively, staining depends on redox status of cells, NF, Red	Oxidation status, compatible with MitoTracker® Green FM [96].
MMP insensitive probes		
Nonyl Acridine Orange, 495/519	Binds cardiolipin in IMS, NF, Green	Mitochondrial mass [97], Toxic at high concentration [98]
Mito-ID Red, 558/690	Aldehyde, Binds cardiolipin, F, Red	Least cytotoxic and phototoxic, superior photostable [47]
MitoTracker Red-580, 581/644	Thiol-reactive, cell permeant, F, Red	Permeabilization with cold acetone for fixation
MitoTracker Deep Red-633, 644/665	Thiol-reactive, cell permeant, F, Red	Permeabilization permits maintaining the staining pattern, Multi-parametric sperm assessments [84]
MitoFluor Red 589, 588/622	F, Red	Useful for both live and fixed cells
MitoTracker Green FM, 490/516	Binds free thiol group, without Washing not required for Fluorescence, NF, Green	More photostable than R123, used for measuring mitochondrial mass and morphology [99]
MitoFluor Green, 489/517	Does not contain chloromethyl moiety, NF, Green	Compatible with dual staining protocols

IMS, Inter-membrane space; Mitochondrial membrane potential ($\Delta\Psi$m); F, fixable; NF, non-fixable, TMRE, Tetramethylrosamine ethyl ester; TMRM,Tetramethylrosamine methyl, NAO, 10-*N*-nonyl acridine orange.

3.8. Mitochondria in apoptosis

Programmed cell death or apoptosis is an evolutionary-conserved physiological mechanism to remove cells from an organism. Mitochondria contain several pro-apoptotic molecules that activate cytosolic proteins to execute apoptosis, block anti-apoptotic proteins in the cytosol and directly cleave nuclear DNA. Mitochondria trap these pro-apoptotic proteins and physically separate pro-apoptotic proteins from their cytoplasmic targets. Apoptosis is then initiated by the release of mitochondrial pro-apoptotic proteins into the cytosol. This process seems to be regulated by Bcl-2-like proteins and several ion channels, in particular the permeability transition pore (PTP) that is activated by almost all pro-apoptotic stimuli [72,73,74].

3.9. Mitochondrial dysfunction and disease

Alterations in mitochondrion integrity are considered as an important factor in a range of human diseases; however, investigations to elucidate the role of mitochondrion in animal diseases have not gained momentum so far. Nonetheless, we list here some of the diseases associated with mitochondrial dysfunction. In humans, several researchers have shown involvement of mitochondrial disorders in autism, multiple endocrinopathy, myopathy, diabetes, Barth's syndrome [74]; Friedreich's ataxia, hereditary spastic paraplegia, Wilson's disease [75]; bipolar disorder, dementia, Alzheimer's disease [76], co-enzyme Q10-deficiency [77]. Moreover, mitochondrion has also been implicated in the aging process [78]. Few cases of mitochondrial involvement in animals have been reported [79–82]. In veterinary practice, mitochondrial myopathy in a German shepherd dog was reported [83].

4. Probes for measuring mitochondrial function

Rhodamine 123 (R123) was the original fluorescent probe to selectively stain functional mitochondria. This probe only fluoresces red when the $\Delta\Psi$m over the IMM builds up. Conventional dyes such as tetramethylrosamine and R123, although readily taken up by functional mitochondria, are subsequently washed out of the sperm cells once the $\Delta\Psi$m is lost [47]. This characteristic limits their use in experiments in which sperm cells must be treated with aldehyde-based fixative or other agents that affect the energetic state of mitochondria. Moreover, results and interpretation of mitochondrial function studied in their native (live) state differs greatly from that of investigations carried out either on mechanically isolated mitochondria or detergent-permeabilized cells. This is due to the role played by cytosol as external medium on the mitochondrial function. To overcome the problem the fluorescent probes (MitoTrackers®, Molecular Probes, Eugene, Oregon, USA) were developed. Using MitoTracker® dyes, stained sperm suspensions can be fixed

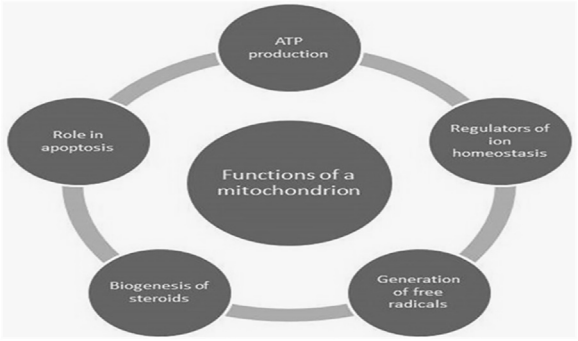

Figure 2. Vital functions of mitochondria.
Mitochondria is credited with performing several important cellular functions *viz*. energy homeostasis and cellular metabolism [13], [50] and [32]; ion homeostasis, including calcium-evoked programmed cell death (apoptosis, [52]) and heme synthesis reactions [53]; generation of free radicals and signaling mechanism [54]; biogenesis of steroids [33] and hormonal signaling [56] and [57]; and role in apoptosis [58]. However, some of the mitochondrion perform functions specific to the cells they inhabitat *e.g*. in liver cells they contain enzymes that allow them to detoxify ammonia, a waste product of protein metabolism.

with mitochondria retaining the stains. They selectively label the respirating mitochondria in live cells and therefore, are suitable for multi-parametric sperm assessments [84]. Reduced MitoTracker® CMTMRos and CMXRos (also called MitoTracker® Orange and MitoTracker® Red, respectively) belong to the rosamine molecule family. They possess the advantage of being retained by the cell after formaldehyde fixation. Their orange or red fluorescence makes them suitable for multicolor protocols. However, MitoTracker® Orange CMH_2TMRos and MitoTracker® X-Rosamine $CM\text{-}H_2XRos$ fluorescence after oxidation, a process that only takes place under oxidative respiration. Since this process is only relevant in functional mitochondria, these probes are suitable to discriminate sperm with deteriorated mitochondria in apoptotic cells from that of aerobically capable living-sperm [85]. Another probe, MitoTracker® Red CMXRos was used to investigate and classify cryo-damaged sperm cells into structurally normal, mid-piece droplet, flagellar droplet, spermatid and others (including head deformities, malformation of the mid-piece or flagellum) [86]. On the other hand, some of the fluorescent probes like JC-1 change their fluorescent properties depending on the changes in the $\Delta\Psi m$ of the inner membrane. Thus, JC-1 switches from orange fluorescence in the aerobic functional mid-piece towards green fluorescent after IMM depolarization [87], and thus can be used to report depolarization of the inner membrane as an indicator of mitochondrial functionality. Smiley *et al*. [9] have suggested JC-1 as the most appropriate choice for assessing $\Delta\Psi m$. In support of above observation, while evaluating various mitochondrial-specific dyes Garner *et al*. [88] found the differential staining of JC-1 to provide a more rigorous estimate of metabolic function than R 123 or Mito-Tracker®. $DiOC_6(3)$, a carbocyanine dye from the DiOC family, cannot be used exclusively for measurement of $\Delta\Psi m$ in intact cells, except after dissipating the plasmatic and ER membrane potentials. To produce rigorous and reproducible results, the dye and the cell concentrations have to be monitored with care. When $DiOC_6(3)$ is used at low concentrations (10–20 nM), this dye rapidly reaches equilibrium in the mitochondria with low quenching effects. The use of higher concentrations (more than 50 nM) may result in non-mitochondrial staining (plasma membrane and endoplasmic reticulum) and in fluorescence quenching [89]. It would be appropriate to mention here that no published results have validated an absolute quantification of $\Delta\Psi m$ from the fluorescence intensities to the expressed values in millivolts; therefore, semi-quantitative fluorescent approach is applied (Refer Table 2 for relative merits of fluorescent probes).

5. Probes for measuring free radicals in mitochondria

Reactive species are continuously generated in normal cellular processes and are essential to continuation of life. Free radicals are produced in the mitochondrial electron transport chain by electron reduction of molecular oxygen by electron donors such as flavor-proteins and semi-ubiquinone species [47]. Free radicals are detrimental to spermatozoa health once homeostasis mechanism is disturbed. Therefore, measurement of reactive species in sperm mitochondria is considered a valuable tool to predict quality. However, investigator must realize that in addition to mitochondrial origin, free radicals from other sources may contribute to their presence in the cytosol.

In addition to very short life, presence of several antioxidant systems in the living spermatozoa makes detection of free radicals difficult. However, fluorescent probes, due to their excellent sensitivity are useful tools to detect sperm free radicals (reviewed by [100]).

Table 3
Salient features of fluorescent probes to measure free radicals species.

Fluorescent probe/excitation/emission spectra ($\ddot{e}_{ex}/\ddot{e}_{em}$)	Radical species detected	Salient features
DAF-FM, 495/515	NO	Cell-permeant and diffuses passively into sperm
Amplex Red, 560/590	H_2O_2 in presence of HRP	Highly specific and sensitive [102] Use in isolated mitochondria, addition of SOD enhances fluorescence [47] Unstable and photosensitive [103]
OxyBURST Green-H_2DCFDA, 504/529	H_2O_2, HOO^-, $ONOO^-$ and HO^-, O_2^-	pH dependent and cellular efflux reduces application, not a reliable probe [104]
Dihydroethidium (DHE), 355/420	O_2^-	Difficult to assess intracellular O_2^-, overlapping fluorescence affects observation [105], careful selection of wavelength is requiredforoptimum results [106]
Mito Sox, 510/580 (modified DHE)	O_2^-	Live sperm permeant, rapid and actively enters mitochondria [47], overlapping fluorescence affects observation [105], Is a modified DHE
Dihydrorhodamine RhH_2, 505/534	H_2O_2 $ONOO^-$	Not dependent on pH [47], Can be used only as a non-specific indicator of intracellular free radicals [107]

Dyes that are stable in a reduced state (non-fluorescent), but that can be oxidized (highly fluorescent) by the species of interest, are widely used as probes for free radical studies. The peroxidase-coupled oxidation of a probe results in the formation of a fluorescent product and defines the principle of quantitatively determining H_2O_2 formation in biological samples such as isolated mitochondria [101]. Table 3 provides useful information in selecting a fluorescent probes to detect sperm free radicals.

6. Future scope

Despite the fact that great advances have been made in elucidating the functional role of mitochondria in health and diseases in humans and application of various probes in measuring mitochondrial activity, similar investigations in animal diseases are far and few in between. Besides the role of mitochondria being power house of cells, new emerging data show their role as a fundamental platform in cellular signaling, with pivotal roles in processes such as cell proliferation, differentiation, autophagy, and cellular immunity. Moreover, mitochondrial pathological signaling can cause disease irrespective of the energy output. Spermatozoa are typical cells with their own mechanism of movement. In this context, it would be interesting to know the functional role mitochondria play in male reproduction and mitochondria-generated free radicals play in male infertility like teratozoospermia or azoospermia, in animal models as well as in human counter parts. What about mitochondrial stress signaling? How to decipher signals for acute and chronic stress in male breeding bulls? And, more specifically, can advances in mitochondrial science help us in discriminating between bulls of high- or low-genetic merits? What about role of mitochondria in various metabolic and other animal diseases? Presumably, a lot remains unanswered and investigators working in veterinary science need to carry out a lot of work to uncover mystery of mitochondrial involvement in infertility and in metabolic aberrations. The aim of this review is to present concise information on mitochondrial science with special reference to selecting probes for measuring mitochondrial parameters including free radicals determination. Addressing these questions would probably bring a new paradigm to male infertility as well as 'mitochondrial dysfunction' specially in metabolic diseases in animal science.

Thus, in line with the above thought process, this article reviewed features and functions of mitochondrion and application of several fluorescent probes employed to evaluate the mitochondrial compartment by measuring its activity. However, questions regarding their specificity and sensitivity in biological samples remained yet to be answered comprehensively. Solaini and co-workers [108] have described different methods using fluorescent probes to assess mitochondrial compartments in living or detergent-permeabilized cells or isolated mitochondria. Despite application of best available fluorescent probe, many errors such as interference with cell or mitochondrial metabolism, photo-induced damages, and probe binding may occur [109,110]. Moreover, staining with fluorescent probes induces an inhibition of mitochondrial respiration, depending on the probe and its concentration. It is important to use low amounts of fluorescent probes to avoid the problems related to their toxicity. For these reasons, the morphological and functional heterogeneity of mitochondria must be taken into account in the interpretation of data. Although electrochemical electrodes were recently developed to monitor NO production [111], the results appeared difficult to reproduce.

Wardman [100] noted that "The ideal chemical probe would be highly reactive at low concentrations: specific, sensitive, without other reactivity, non-toxic, well characterized chemically, easy to load into organelles, cells or tissues without subsequent leakage or unwanted diffusion, excretion or metabolism, readily available, easy to use without specialized apparatuses and cheap".

Conflict of interest statement

The authors declare that they have no conflict of interest.

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