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Structural, Physiological, Biochemical and Biotechnological Aspects of Mammalian Spermatozoa: An Overview

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Abstract
Fertilization, an important phase of reproduction, is physiologically the union of the male and female gamete. This progression involves the fusion of an oocyte with a sperm, leading to generation of a single diploid cell, the zygote, from which a new individual organism develops. The elucidation of the physiological, biochemical and molecular mechanisms of fertilization has enthralled researchers of relevance for several years. This overview embodies this fascinating progression at physiological, biochemical and molecular level. A number of molecules have been recognized to play a key role in each step of this interesting process pertaining to the sperm magnetism from the oocyte, the sperm maturation, the sperm and oocyte union and the two gamete pronuclei fusion leading to the development of zygote. The compilation of data and salient hypotheses covered by this review provides new insights into perception of the causes of fertility issues due to fertilization disorders using the platform of *in silico* studies.

Keywords: Fertilization, Zygote, Gamete fusion, Sperm capacitation, Cortical reaction

Introduction

An individual living system has a restricted lifetime. Though, through fertilization all living organisms are able to continue life as a species. The major role of spermatozoa is to fertilize eggs (1). Conversely, mammalian spermatozoa cannot achieve this task as ejaculated. They must foremost undergo a physiological change known as capacitation followed by a morphological change recognized as the acrosome reaction in the female reproductive tract. Spermatozoa also port the capability to wander into the oviduct, where they act together with and subsequently fuse with the egg. A number of factors, which contribute to sperm-egg interactions have been identified (2), based on observations employing enzyme inhibitors and antibodies in *in vitro* fertilization systems. It led to the conclusion that various sperm enzymes within the acrosome dissolved the egg components and that different membrane proteins were used for binding with eggs. However, recent experiments by means of gene disruption of these factors did not result in an infertile phenotype, suggesting that they are not essential for fertilization, although they may indeed play a role during the fertilization event (3). In contrast, employing *in vivo* gene-targeting experiments, a number of proteins have unexpectedly emerged as being essential factors for fertilization (4,5). Certain arising views on mammalian spermatozoa function and interaction with egg i.e., fertilization is reviewed and compared with previously postulated models as follows:

1. Spermatogenesis

Spermatogenesis is a process in which testicular stem cells develop into mature spermatozoa, having subcategories as: (a) spermatocytogenesis (mitosis), (b) meiosis and (c) spermiogenesis. These developmental events occur in the seminiferous tubules of the testes. Spermatocytogenesis is a series of mitotic divisions that starts with diploid (2n) spermatogonia (A1) cells, which are the most immature male germ cells. Spermatogonia are located at the periphery of the seminiferous tubules, and as they mature, move towards the lumen of the seminiferous tubules. The end results of spermatocytogenesis are primary spermatocytes, which remain as 2n cells. The purpose of spermatocytogenesis and the series of mitotic divisions are to provide base cells that will ultimately become spermatozoa (sperm). The spermatogonia serve as a pool of stem cells that allows the process of spermatogenesis to continue indefinitely (6). The production of gametes in the male only occurs by meiosis in the testis. The final step of meiosis separates homologous chromosomes to form two 1n diploid cells. During the process of meiosis DNA is synthesized, duplicated and divided. The final step of the original spermatogonia is the metamorphic transformation to a 1n sperm cell. The sole purpose of sperm is to transport the male's DNA to the site of fertilization in the female. To accomplish this goal, round spermatids need to physically change in shape so that

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their new shape will allow them to be progressively motile and progressively motile and move in a forward motion. This final step of metamorphism is the final testicular product that is the tadpole-shaped sperm that society is familiar with, head, neck and tail. When the process of spermatogenesis is complete sperm will leave the testes and enter the male reproductive tract where they will complete the process of maturation (7). The process of spermatogenesis is a very productive and efficient way of producing a large number of sperm capable of fertilization. It has comprehended that the incidence of abnormal sperm is basically an error in the process of spermatogenesis (8). However, according to a research study, abnormal sperm were purposefully created and were never meant to partake in the act of fertilizing an ovum, but rather, they were constructed for the purpose of sperm competition and to hinder other male's sperm from achieving fertilization in instances when a female mates with multiple males during estrus (9).

2. Functions of the epididymis

The epididymis performs various major functions necessary for the reproduction of most mammalian species. First, the epididymis serves as a passage way for sperm to travel into the vas deferens. The epididymis also concentrates the sperm, provides the environment and fluids necessary for sperm maturation and acts as a storage unit for immature sperm that are ready for ejaculation. As sperm leaves the testicle it is accompanied by rete testis fluid, which is a diluent that makes ductile transport of the sperm easier.

Upon reaching the epididymis, epithelial cells absorb rete testis fluid and secrete epididymal fluid, which concentrates the sperm and allows for more storage space (10,11). While the sperm travel from the caput to the cauda epididymidis they continue to go through maturation. This maturation involves the migration of the cytoplasmic droplet, located on the mid-piece to travel from the proximal position to the distal position.

Also, while moving through the epididymal duct; rat sperm acquire the ability of increased progressive motility (12,13). Maturation of sperm predominantly takes place while sperm move from caput to the corpus sections of the epididymis (12,14).

It has been reported that sperm from all regions of the epididymis have diverse levels of motility, with the percentage of motile cells and the progressive swimming pattern of these cells being higher in samples closer to the cauda epididymidis rather than the caput epididymidis (14). There is evidence that upon leaving the corpus, sperm are bound with "forward motility protein" (FMP), which allows the caudal sperm to move progressively rather than in circles or thrashing, which is observed in sperm from the caput region (15). FMP is attached to epididymal sperm just prior to entering into the proximal epididymis. It is believed that FMP is activated with elevated levels of cyclic AMP to produce progressive motile sperm. The mechanism by which FMP and cyclic AMP act is not yet understood (15). It is thought that the process by which sperm attains its capability to fertilize is due to the addition and/or subtraction, or altering of surface proteins, until the correct receptors are on the surface of the sperm. These sperm receptors permit same-species recognition between the sperm cell and the oocyte. The exact mechanisms, which maintain potentially motile mature sperm in a quiescent state, are not completely understood. However, the duration that the epididymis can maintain viable sperm is variable between species.

Most researchers agree that the purpose for sperm being maintained in an immotile quiescent state is to conserve energy stores and postpone cellular membrane reactions, which will be required when the sperm travel through the upper female reproductive tract. However, there is debate over the mechanism in which sperm are maintained in their quiescent state. Some evidence suggests that sperm are held in quiescence by a change in osmotic pressure, since sodium and chloride ions are in much lesser concentrations in the epididymal fluid than they are in circulating blood (2,16). Also, it has been speculated that amino acids could play a role in maintaining epididymal sperm immotile. This speculation is based on reports that in the caudal epididymal plasma, the amino acids glutamate, glutamine and asparagine are found at a higher concentration in the ram (2,17) and glutamine in the bovine (12).

A high molecular weight protein (>200 kd), located in the caudal epididymal fluid of the rat epididymis has been well described (14). Since the presence of this protein had not been reported previously, they named this protein, "immobilin". It was proposed that due to the high molecular weight of this protein, it made the caudal fluid more viscous and physically inhibited sperm motility.

3. Comparison of epididymal sperm and ejaculated sperm

The ultimate goal in reproduction is to produce pregnancies and the method that will produce the best results is always going to be natural mating or at least the use of ejaculated semen. However, in many cases natural mating is not an option and ejaculated semen is unavailable, due to difficulty of handling the animal, death prior to collection or obstructive azoospermia preventing ejaculation (18). In these cases, the best alternative source of viable, reproductively capable sperm is those stored in the cauda epididymidis. Research has shown that cauda epididymal sperm, when used with AI can produce offspring in a multitude of species, for example, the eland antelope (19), goat (20), dog (21), gaur (22) and Spanish ibex (23). When used with ICSI epididymal sperm has produced offspring in a few species including cattle (24) and rats (25) reported that using ICSI with epididymal sperm on in vivo or in vitro matured pig oocytes can result in cleavage, however, using fresh ejaculated sperm produced significantly higher cleavage rates than did epididymal sperm. More recently, Umeyama et al. (26) reported the birth of piglets using ICSI and epididymal sperm.

Despite its obvious reproductive potential, epididymal sperm does have some characteristics that make it noticeably different from ejaculated sperm. The most noticeable difference between epididymal and ejaculated sperm is the cytoplasmic droplet. This droplet can be located anywhere along the mid-piece of the sperm. However, maturity is estimated by the location of the droplet, the more distal the droplet is from the head the more mature the sperm. The cytoplasmic droplet has been well described (27) as remnant of the cytoplasm from when the maturing cell was a spermatid. The shedding of the cytoplasmic droplet occurs when the sperm are introduced to seminal fluid.

Other than the physical differences, there are also metabolic differences between epididymal and ejaculated sperm (28). Epididymal sperm respire at a much slower rate than ejaculated sperm, and from this observation it was concluded that epididymal sperm is more efficient when it comes to the oxidative generation of utilizable energy (28). It has been reported in Red deer, that although post-thaw motility of epididymal sperm is equal, 8% glycerol is recommended over 4% glycerol due to the better post-thaw acrosomal protection (29). Epididymal and ejaculate sperm have been reported to respond differently to caffeine when used as a sperm motility stimulant (30). When equine epididymal sperm were incubated in a medium with caffeine, the sperm motility improved over time, however, when ejaculated sperm were incubated in the same medium their motility decreased over time. It was hypothesized that since the epididymal sperm were never coated with seminal plasma components, their susceptibility to caffeine was increased. It was also proposed that caffeine might inhibit cyclic nucleotide phosphodiesterase more efficiently in epididymal sperm, which would increase the levels of cyclic AMP of the epididymal sperm (31).

The region from which sperm are retrieved is crucial to its fertilizing potential. In the ram, it has been shown that sperm harvested from the corpus or caput epididymidis were unable to penetrate hamster oocytes *in vitro*. While sperm from the cauda epididymis penetrated hamster oocytes at a rate that was not significantly different from that of ejaculated sperm (32).

These subtle but obvious differences can all be attributed to the environment or fluid in which the sperm are located (e.g., seminal plasma for ejaculated, epididymal fluid for epididymal sperm). Epididymal plasma has many factors that maintain epididymal sperm through mechanisms that are not yet understood. There are a multitude of components in seminal fluid including: citric acid, ergothioneiene, fructose, glyceryl phosphorylcholine, sorbitol, ascorbic acid, amino acids, peptides, proteins, lipids, fatty acids and numerous enzymes (33-35).

These chemical elements found in seminal fluid differ between species due to size and absence of different accessory glands. Even though there is a wide array of research on mammalian seminal fluid, there are conflicting theories about whether seminal fluid helps or hinders sperm. For example, it has been reported that when epididymal sperm was collected into prostatic fluid of the dog there were less sperm possessing a pre-freeze cytoplasmic droplet (21). Also, the dog epididymal sperm had higher post-thaw motility and viability values in those sperm that were exposed to prostatic fluid compared to those that were not exposed to prostatic fluid (21).

Furthermore, the dog epididymal sperm in the prostatic fluid treatment showed a higher motility value after 6 hours at 20°C. Similar to the dog, a study in Red deer showed that allowing epididymal sperm to incubate and then freeze with seminal plasma improved post-thaw viability and motility values of the epididymal sperm (29). Also, has been documented that when seminal plasma is introduced to epididymal bovine sperm, and then removed, the occurrence of cytoplasmic droplets was reduced. The reduction of the droplets was thought to improve morphology of the epididymal sperm post-thaw since it was believed that the cytoplasmic droplets had a negative effect on the sperm morphology when subjected to cryopreservation. Moreover, it was shown that when African buffalo epididymal sperm were frozen with bovine seminal plasma the results were significantly less than when sperm was not exposed to seminal plasma (36).

Whether or not seminal plasma is beneficial to sperm, it does apparently serve a physiological purpose. It has been demonstrated in the bull that seminal plasma indirectly assists in the initiation of sperm capacitation, which is necessary to complete fertilization (37-39). It has been reported that exposure of Murrah buffalo epididymal sperm to isolated seminal plasma proteins (heparin and gelatin binding) assisted the sperm in mucous penetration and protected sperm membranes *in vitro* (38). Furthermore, the concentrations 20, 30 and 40 mg of heparin and 30 and 40 mg of gelatin binding proteins showed better membrane protection than 10, 50 and 60 mg. All protein treated epididymal sperm showed a significantly higher membrane protection than did the control.

4. Limitations in the use of epididymal sperm

Even with some success in the field of assisted reproduction, epididymal sperm has limitations that include: methods for harvesting sperm from the epididymis, techniques for freezing epididymal sperm, the cytoplasmic droplet and in most cases, collection of epididymal sperm is from a postmortem animal.

There are three main methods being used to collect epididymal sperm. However, depending on the laboratory there are subtle variations in these methods. With each method the epididymides along with the vas deferens are dissected away from the testicle. The first method is mincing or dicing the epididymis up while it is in a sperm medium. In the medium the sperm will then swim away from the tissue and be collected by pipette or filtration (40). This method is often used in smaller species where the epididymides are difficult to manipulate due to their small size (41). The second method is called the slicing, or puncture, method. In both the first and second method, much of the connective tissue and superficial blood vessels are dissected away from the epididymis. The epididymal ducts are either cut with a scalpel blade or punctured in several places with a needle. After the incisions the epididymis is often milked, to extract as much sperm as possible. Again, the epididymides are placed into a medium and allowed to incubate so the epididymal sperm can swim up into the medium and away from the epididymal tissue. The sperm rich medium is collected by either filtration or pipette (19, 42).

The third method of epididymal sperm collection is the retrograde flush method, which has been reported in the stallion (43), African buffalo (36, 44) and Red deer (45). The idea of the retrograde flush is to move the epididymal sperm in a direction opposite of its normal transport. For this method, the vas deferens is threaded with either a needle or small tube, both of which are attached to a syringe, and then, either medium or air is used to push the sperm out of single small incision that was made at a distal location in the cauda epididymidis.

It has been shown in Red deer that the flushing method is preferred over the slicing method, because the flushing method produced less contamination and higher quality sperm (41). Even though this particular project showed no difference in concentration, the authors believed that if executed correctly, flushing could produce higher sperm numbers than the slicing method. Also It has been stated that the flushing method was an acceptable method to use out in the field when collecting epididymal sperm from African buffalo (46). In contrast, a study showed no difference in sperm concentration, pre-freeze total and progressive motility or morphology between the flushing method and a modified slicing method known as flotation method, when collecting epididymal sperm in the stallion (28).

Due to the lack ofdifference between the two methods, they preferred the flotation method because it is easy to prepare (28).

5. Clinical diagnosis and evaluation of seminal oxidative stress

5.1. Spinal Cord Injury

Cetain earlier studies have reported the increased ROS levels in the semen of 25% to 40% of infertile men (2,47). They also documented that in men with spinal cord injury, elevated seminal ROS levels are connected with poor sperm motility as well as morphology. These relations are independent of both ejaculation method and specimen category (47).

5.2. Varicocele

The role of ROS in varicocele has been reported recently (48,49). Extreme nitric oxide release within dilated spermatic veins has been recognized in subfertile males with varicocele. This nitric oxide release may cause spermatozoal dysfunction (50). A positive correlation between seminal ROS levels and varicocele grade with significantly higher levels of seminal ROS are seen in men with varicocele grades 2 and 3 compared to men with varicocele grade 1 (51,52). Varicocele patients also reflect low seminal plasma TAC levels and increased 8-hydroxy- 2'-deoxyguanosine levels, indicative of a deficient prooxidant defense system concomitant with oxidative DNA damage (53,54). According to the study (55), varicocele patients as compared with normal sperm donors have considerably increased oxidative stress parameters such as ROS and lipid peroxidation as well as significantly decreased antioxidant concentrations. Antioxidant supplementation may thus be advantageous to this infertile population with varicocele. Mostafa et al. (56) first reported that varicocelectomy reduces the seminal plasma ROS levels of infertile men associated with increased seminal plasma concentrations of antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and vitamin E of infertile men. Couples who do not achieve pregnancy following varicocelectomy might notably increase their pregnancy and live birth rates after undergoing intrauterine insemination, in spite of failing to illustrate improvements in semen parameters (57).

It is hence recommended that pregnancy rate improvement following varicocelectomy may be because of functional factors such as seminal oxidative stress and the spermatozoal DNA integrity not regularly tested during customary semen analysis (57).

5.3. Leukocytospermia

ROS in the human ejaculate instigate principally from seminal leukocytes. is characterized by Also, abnormally high seminal leukocyte, polymorphonuclear neutrophils and macrophages are potential biochemical markers of leukocytospermia (58). Seminal leukocyte ROS production has been reported to induce spermatozoal damage during ART trials (59). Patients with accessory gland infection display both leukocytospermia and elevated ROS levels (60,61). In these patients, sperm function defects are consequential of abnormal lipid peroxidation, triggered by the elevated ROS levels (62).

5.4. Genito-Urinary (GU) Tract Infection

Throughout GU infection, the presence of leukocytes in semen has been coupled with decreased sperm motility and fertilization capacity (63,64). However, Khan et al. (65) reported no connection between standard seminal parameters and leukocyte concentration in human semen). This dilemma may be partially because of the different techniques used to find out leukocyte concentration in semen as well as the lack of conformity on the lower leukocyte concentration accountable for sperm damage (63,66). Infections sited in the testis and epididymis generate ROS, which are particularly harmful to spermatozoa due to its lack of a pro-oxidant resistance system. Sperm function may also be indirectly exaggerated by an infection exciting the presence of ROS in the prostate gland and seminal vesicles. An association between prostatitis and male infertility has been reported, but the responsible mechanism is still poorly understood. Prostatitis is associated with the presence of granulocytes in prostatic fluid. Irrespective of leukocytospermia status, increased seminal oxidative stress is reported in men with chronic prostatitis and prostatodynia (67). Such findings support the controversial prostatitis-infertility association debate. Numerous hypotheses converse male genital tract infections and their connection with ROS. Particularly, the leukocytes stimulate human spermatozoa to produce ROS. The mechanisms accountable for such excitation are still obscure, but may consist of the direct contact of sperm and leukocytes or may be regulated by leukocyte release of soluble products (68,69).

5.5. Environmental Factors

A relationship between cigarette smoking and reduced seminal quality has been recognized (70). Injurious substances including alkaloids, nitrosamines, nicotine, cotinine and hydroxycotinine are known to be present in cigarettes and produce free radicals (71,72). In a study, Saleh et al. (73) compared infertile men who smoked cigarettes with nonsmoker infertile men. It could be inferred that infertile men who smoke cigarettes show higher seminal OS levels as compared to infertile nonsmokers, probably consequent to considerable enhancement in leukocyte concentration in their semen. Notably higher levels of DNA strand breaks in men who smoke have also been recognized. DNA strand breaks may be consequential from the presence of carcinogens and mutagens in cigarette smoke. In recent years evidence evocative of the injurious effects of occupational exposure chemicals identified as endocrine disruptors on the reproductive system has progressively accumulated (74).

Environmental pollution is a key source of ROS production and thus has been concerned in the pathogenesis of poor sperm quality (75,76). Tollgate workers with continuous environmental pollutant exposure had inversely interrelated with blood methaemoglobin and lead levels to sperm parameters in comparison to local male inhabitants not exposed to comparable automobile pollution levels. These findings suggest that nitrogen oxide and lead, both present in the composition of automobile exhaust, adversely affect semen quality (77,78). Besides, the increase of industrialization has been reported to consequent an elevated deposition of highly toxic heavy metals into the atmosphere. Paternal exposure to heavy metals such as lead, arsenic and mercury is linked with decreased fertility and pregnancy delay (79-81). Oxidative stress is hypothesized to play a pivotal role in the progression of adverse health effects resultant of such environmental exposure (82,83).

6. Free radicals and assisted reproductive procedures

Several conditions related with male infertility, e.g., microdeletions of the Y chromosome, sperm maturational arrest, meiotic defects, aneuploidies, defective centromeres and defects in oocyte activation still lack a definite treatment. Though, advances in ART have assisted in improving management of male factor infertility (84). ICSI is the most common ART technique, while it is connected with the quality after ejaculation is density gradient centrifugation, sedimentation, glass wool filtration and conventional swim-up (85). The first three preparation techniques are more efficient in reducing levels of free radicals than the conventional swim-up technique (85). Besides, high ROS levels are coupled with decreased pregnancy rate following IVF or ICSI and arrested embryo growth. Based on a metaanalysis that included all of the available evidence from the literature, there is a noteworthy relationship between ROS levels in spermatozoa and the fertilization rate after IVF (86). Thus, measuring ROS levels in semen specimens before IVF may be practical in predicting IVF outcome and in counseling selected patients with male factor or idiopathic infertility.

7. LABORATORY ASSESSMENT OF OXIDATIVE STRESS IN INFERTILITY PRACTICE

7.1. ROS Measurement

For clinical purposes, it is important to have a trustworthy and reproducible technique of ROS measurement. Numerous methods are available to measure ROS levels in semen (87). Direct techniques such as electron-spin resonance spectroscopy, also known as electron paramagnetic resonance, have been applied chiefly for research purposes since these are comparatively expensive technologies, which require fresh samples, and vast technical expertise (88,89). This procedure is used to identify electromagnetic radiation being absorbed in the microwave region by paramagnetic species, subjected to an external magnetic field. This practice is the only analytical approach, which allows the direct detection of free radicals and reports on the magnetic characteristics of unpaired electrons and their molecular environment (90). Nevertheless, short life span of ROS enables the application of these techniques difficult.

Indirect methods, e.g., chemiluminescence methods are usually applied for monitoring ROS produced by spermatozoa. This assay measures both intracellular and extracellular ROS depending on the probe used. Chemiluminescence estimates the amount of ROS, not the level of the sperm-damaging ROS present at any given time. Moreover, it can distinguish between the production of superoxide and hydrogen peroxide by spermatozoa depending on which probe is used. Two probes may be used with the chemiluminescence assay, namely, luminol and lucigenin. A luminol-mediated chemiluminescence signal in spermatozoa appears when luminol oxidizes at the acrosomal level. Luminol reacts with a number of ROS and permits both intracellular and extracellular ROS to be assayed. Lucigenin, though, yields a chemiluminescence likely to be more precise for superoxide anions released extracellularly (91).

The number of free radicals produced is counted as photons per minute. Presence of leukocytes as a confusing factor and the need of fresh semen samples with high sperm count ($>1x10^6$/ mL) are the limitations of this method (91). Furthermore, other multiple factors that affect chemiluminescence consist of the concentration of reactants, sample volume, reagent injection, temperature control, instrument sensitivity, and background luminescence (92). A multiplicity of luminometers is accessible to determine the light intensity consequential from the chemiluminescence reaction. Single/double tube luminometers are insightful and inexpensive however can assess only one or two samples at a given time, which are suitable for small scale research laboratories. In contrary, multiple tube or plate luminometers are more expensive as they can measure multiple samples at the same time and are suitable for centers that are engaged in regular research studies on chemiluminescence (91).

7.2. ROS-TAC Score

Since oxidative stress is caused by an imbalance between levels of ROS produced and antioxidant protection at any given time, it is a possible that assay of oxidative stress can be made either by assessment of ROS or total antioxidant capacity (TAC).) A ROS-TAC score for assessment of seminal oxidative stress has showed to be superior to ROS or TAC alone in discriminating fertile and infertile population (93,94). This score minimizes the inconsistency of the individual parameters (ROS or TAC) of oxidative stress. The ROS-TAC score was based on a group of normal healthy fertile men who had very low levels of ROS. Men with male factor or idiopathic infertility had significantly lower seminal ROS-TAC scores compared to normal controls, or the men with initial male factor that eventually were able to initiate pregnancy. The average ROS-TAC score for fertile healthy men was 50 % that was significantly higher ($p \leq 0.0002$) compared to infertile patient (35.8 %). The probability of successful pregnancy is assessed at < 10% for values of ROS-TAC < 30, however increased as the ROS-TAC score increased.

7.3. Leukocyte Evaluation

While lower leukocyte levels are sometimes linked with considerable ROS levels in semen it is important to determine the exact source of ROS in semen as the clinical implications of sensitive leukocytes are quite different from those of pathological conditions in which spermatozoa themselves are the source of ROS (59,95). Techniques that are presently used for evaluation of seminal OS, such as chemiluminescence assays, do not provide information on the differential contribution of spermatozoa and leukocytes to ROS production in semen. Nitroblue tetrazolium test (NBT) reaction can indirectly reveal the use for detection of seminal oxidative stress, and the differential contribution of cells to ROS creation, and to conclude the state of activation of seminal leukocytes. ROS levels assayed by chemiluminescence assay are strongly interrelated with the results of NBT staining. Besides, the NBT reduction test is generally available, simply performed, inexpensive and has elevated sensitivity (96,97).

7.4. Oxidative Stress Status (OSS)

Presently, there is no agreement regards to the inclusion of ROS measurement as part of the routine clinical assessment of male infertility mostly because of a lack of standardization of ROS analytical techniques, equipment, and range of normal levels of ROS in semen. Some researchers have defined the basal levels of ROS in neat semen specimens of normal healthy donors (98). Assessment of ROS levels in neat semen after liquefaction in the presence of seminal antioxidant protection proved to be a superior test to evaluate oxidative stress status. The ROS levels for fertile donors with normal genital examination and normal standard semen parameters were reported to be 1.5 x 104 cpm/20 million sperm/mL. By means of this value as a cutoff, infertile men can be classified as either OS-positive (> 1.5 x 104 cpm/20 million sperm/mL) or OS-negative (≤ 1.5 x 104 cpm/20 million sperm/mL), irrespective of their clinical diagnosis or data of standard semen analysis (98). Assessing ROS directly in neat semen revealed diagnostic and prognostic capabilities alike to those obtained from ROS-TAC score (98). The antioxidant activity of seminal plasma is removed all through sperm washing steps, also resulting in elevated ROS levels. Extreme washing and manipulation including duration of centrifugation was observed to be more significant than the force of centrifugation for ROS formation by human spermatozoa (99). Consequently, procedures that minimize multiple centrifugations, resuspension, and vortexing should be used for the preparation of spermatozoa for ART (100). Conflicting studies make it difficult to establish the clinical value of ROS measurement in medical practice because there is no clear proof whether elevated ROS levels are a basis or an effect of abnormal semen parameters and sperm injure (101).

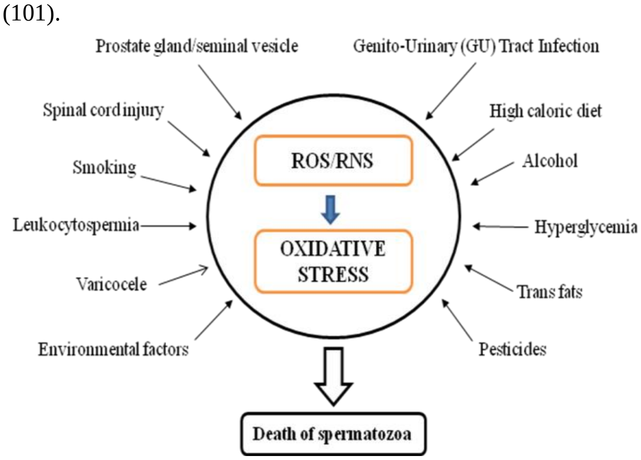

Figure 1. Sources of oxidative stress during spermatogenesis

Taken together the illustrations in Figure1 pertaining to certain sources of oxidative stress versus spermatogenesis, updated relevant findings recommend that ROS measurement should be used as a diagnostic tool in infertile men especially in cases of idiopathic infertility and that the reference values of ROS in neat semen can be used to define the pathologic levels of ROS in infertile men and may guide in better therapeutic interventions.

8. Strategies to reduce seminal oxidative stress

Certain foremost role of oxidative stress in the pathogenesis of male infertility, treatment strategies with the goal of reducing levels of seminal oxidative stress are essential for natural as well as assisted reproductive technologies. Spermatozoa generate small amounts of ROS that must be constantly inactivated to keep only the required amount to maintain normal physiologic cellular function.

The pathologic levels of ROS detected in the semen of infertile men are probably caused by increased ROS production than by reduced antioxidant capacity of the seminal plasma (102,103; Figure 1). The body has a number of mechanisms to minimize free radical induced damage. Regrettably, spermatozoa are unable to repair the damage induced by oxidative stress, because of lacking the requisite cytoplasmic enzyme systems to carry out the repair (60,61). Antioxidants are the most vital defense mechanisms against OS induced by free radicals. It scavenges the rective radicals and demolishing them to become less active, less dangerous. Metal chelators and metal binding proteins, blocking new ROS formation are classified as preventative antioxidants. Endogenous antioxidants defense include enzymatic and non-enzymatic molecules usually distributed within the cytoplasm and various cell organells, namely, vitamins E and C, beta-carotene and other antioxidant dietary supplements, glutathione and enzymes, work through removing ROS already produced by cellular oxidation (104,105). Numerous clinical trials have established the beneficial effect of antioxidants in treating selected cases of male infertility (106- 109), while others failed to account the same benefits (110). The majority of the studies analyze multiple antioxidant combinations, different dosages and durations. Also, the patient's selection is another significant aspect because oxidative stress can not be considered the root of male infertility in all patients. Also, according to an overview published by a team of researchers led to conclusion that many studies suffer from the lack of placebo-controlled, double-blind design, making the effectiveness of antioxidant supplementation in infertile patients still inconclusive (60,61).

9. Origin of dna damage in spermatozoa

Sperm genetic material is ordered in a special fashion that keeps the nuclear chromatin extremely stable and compact. The normal DNA structure is capable of decondensation at suitable time transferring the packaged genetic information to the egg without defects in the fertilization process. The reason of DNA damage in sperm can be recognized to be connected to many environmental conditions including air pollution, cigratte smoking (111,112), pesticides, chemicals, heat and ART prep protocols (76) irradiation (73,113,114), chemotherapy to pathophysiological conditions such as cancer, varicocele (73), high prolonged fever (115), advanced age (68,69) or leukocytospermia (116). Exact molecular mechanisms lead to sperm DNA damage and/or chromatin abnormalities are not fully understood but there are recently three major theories which are related i.e., chromatin packaging abnormalities, reactive oxygen species and apoptosis. Most of these agents not only disorder hormone levels but may also induce oxidative stress, which could harm sperm DNA (117). Nuclear DNA damage was inversely linked with methaemoglobin levels. Infertile men with varicocele revealed considerable higher DNA damage, which appears to be associated with high OS. Normal semen has low DNA integrity and resist to leukocytospermia. Sperm DNA damage is higher in men with cancer even before cancer therapy. Increased ROS production showed a positive correlation with sperm DNA damage in a time-dependent manner. DNA fragmentation was strongly positively correlated with intrinsic ROS production, whereas this correlation was weaker for extrinsic ROS production.

The level of sperm DNA damage has been strongly coupled with impaired sperm function as well as male infertility. Though the precise mechanism(s) responsible for chromatin abnormalities in human spermatozoa is/are most likely to be multi factorial and are not accurately understood at this time. The most significant theories proposed as molecular mechanism of sperm DNA damage are: (a) defective chromatin packaging (47), (b) reactive oxygen species (ROS) (47), (c) apoptosis principally during spermatogenesis (87,118), and (d) DNA disintegration induced by endogenous endonucleases (119).

10. Role of oxidative stress in sperm dna damage as associated with male infertility

Extreme generation of ROS in the reproductive tract not only affect the fluidity of the sperm plasma membrane, however also the integrity of DNA in the sperm nucleus. DNA bases are susceptible to oxidative damage ensuing in base alteration, strand breaks, and chromatin crosslinking. Oxidative stress-induced DNA damage causes pro-mutagenic transformation that in its most severe form affects the quality of the germ line and prevents fertilization. When there is less oxidative damage, fertilization can occur, although the oocyte must repair the DNA strand breaks before the initiation of the first cleavage. Apoptosis and ROS are concerned with mediating DNA damage in the germ line (86). The Y chromosome is predominantly susceptible to DNA damage because of its genetic structure as well as it cannot correct double-stranded DNA deletions.

Fertile healthy men with normal seminal parameters almost consistently have low levels of DNA breakage, while infertile men, in particular those with abnormal seminal parameters (Figure 2), have higher fraction of sperm DNA damage (120). Idiopathic infertile men may present standard routine seminal parameters (concentration, motility, and morphology) with abnormal DNA integrity (120, 121; Figure 2). It is of great apprehension that the most competent ART techniques used to treat male factor infertility with high level of sperm DNA damage. During ICSI, it is always advantageous to select spermatozoa with normal morphology, reduceing the risk of introducing spermatozoa with strand breaks (122). This is occasionally not always true because the traditional sperm parameters, namely, sperm count, motility and morphology, have been proven to be poorly interrelated to DNA damage status (123; Figure 2). Furthermore, this has noteworthy clinical implications because *in vitro* fertilization using spermatozoa with damaged DNA may lead to paternal transmission of defective genetic material with adverse consequences for embryo development.

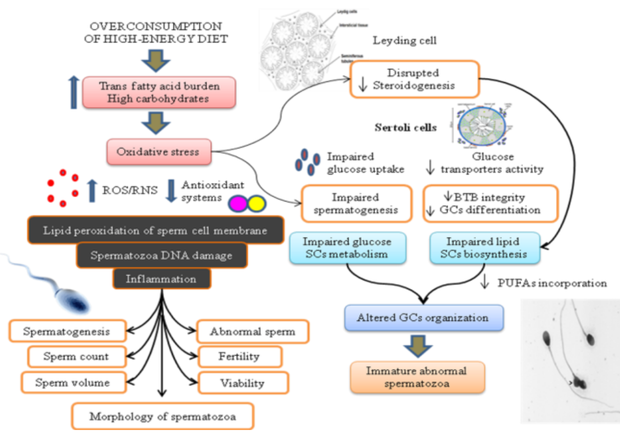

Figure 2. Impact of ROS/RNS on integrity of spermatozoa

These findings recommend that an estimate of the percentage of DNA damaged spermatozoa in fertile and infertile men may be significant and a future challenge will be to develop methods to recognize and select spermatozoa with intact DNA during the IVF/ ICSI procedures. Sperm from infertile men with varicoceles have been connected with considerably high levels of DNA damage (124). The finding of high seminal OS in patients with varicoceles may point out that OS plays a key role in the pathogenesis of sperm DNA damage in patients with this condition. Though varicocelectomy can improve human sperm DNA integrity in infertile men with clinical varicoceles (125); an inadequate number of studies have examined potential treatments to decrease sperm DNA damage. Therapeutic conditions have been recommended that avoidance of gonadotoxins (126); smoking, medications and hyperthermia (127,128); saunas and hot tubes may reduce sperm DNA damage (128). Treatment of GU infection can also be supportive based on the proof that leukocytospermia induce ROS production and perhaps DNA damage. It has been well recommended that sperm DNA damage can be reduced with oral antioxidants administered during a reasonably short time period (129).

11. Evaluation of sperm chromatin integrity

A number of techniques can determine DNA defects in human spermatozoa and the capability of these techniques to precisely estimate sperm DNA damage depends on many technical and biological aspects. Conversely, to establish a threshold level between the fertile population and the lowest sperm DNA integrity requisite for achieving pregnancy remains enormously challenging. Currently both direct (fragmentation, oxidation) and indirect (sperm chromatin compaction) methods are available to assess the integrity of sperm DNA. Direct methods for detecting DNA breaks include (a) the single-gel electrophoresis assay (Comet assay) and (b) terminal deoxynucleotidyl transferase-mediated 2`-deoxyuridine 5`-triphosphate (dUTP)-nick end-labeling (TUNEL) assay (130,131). Indirect methods principally sperm chromatin integrity assays (SCSA) for assessing DNA damage uses chromatin and/or DNA intercalating dyes such as acridine orange to distinguish single-stranded and double-stranded DNA (132,133) . Less frequent clinical tests for DNA damage consist of the sperm chromatin dispersion test (SCD) using the Halosperm kit, allowing to simultaneously perform DNA fragmentation and chromosomal analyses in the same sperm cell (134), liquid chromatography that detect oxidized DNA nucleotide residues (135,136) and evaluation of nuclear protein (protamine/histone ratio) levels in sperm samples.

All techniques presently lack a threshold, except for the sperm chromatin structure assay (SCSA), which assesses the ability of the DNA to resist denaturation by acid or heat and uses DNA flow cytometry approach. The sperm DNA damage is articulated as the DNA fragmentation index (DFI) (137), which can differentiate fertile and infertile population in clinical practice.

12. DNA damage and reproductive outcome

Sperm DNA damage is critical in the context of success of assisted reproductive techniques (55,138). The major nuisance of ART is that they bypass the natural defense barrier present all the way through female reproductive tract accountable for

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selecting the best spermatozoa for oocyte fertilization. Generally, oocytes are capable of repairing partial DNA damage. However, when the damage is severe, embryo death and miscarriages are more likely to happen. Probably that explains why miscarriage rate is higher after ICSI compared to classic IVF (139,140). Standard semen parameters do not recognize subtle defects in sperm chromatin architecture that after the advent of ICSI has become more significant parameter of sperm functional quality than count, motility or morphology. The stress on assessment of genomic integrity has recently increased due to reports that correlate the level of DNA damage with various fertility indices including rates of fertilization, embryo cleavage, implantation, pregnancy and live birth (141).

Sperm DNA integrity is an indispensable requirement to achieve pregnancy in natural conception as well as for IVF outcomes where the natural process of fertilization is circumvented (142). A high level of sperm DNA damage has been found in couples presenting with unexplained recurrent pregnancy loss (143). All male partners of couples who achieved a pregnancy during the first three months attempting to conceive had < 30% sperm with fragmented DNA (144), while, 10% of the couples who achieved pregnancy in months 4-12 and 20% of couples who never achieved a pregnancy had > 30% sperm with fragmented DNA. Furthermore 84% of the men who initiated pregnancy before three months had sperm DNA damage levels of < 15%. Also, for IUI, there was a considerably higher possibility of pregnancy/ delivery in the group with DFI < 27% and HDS (extremely DNA stainable) of < 10% than in patients with DFI > 27% and HDS > 10%. Though, no statistical distinction between the outcomes of IVF versus ICSI was noticed in the group with DFI < 27%, ICSI had notably better results than those of IVF in patients with DFI > 27%. It was concluded that combining the two SCSA parameters, DFI and HDS is a useful method for prediction of IUI outcomes.

Even though sperm DNA fragmentation did not link with the fertilization and embryo fragmentation rates, patients with an elevated percentage of TUNEL positive spermatozoa (> 36.5%) revealed a considerably lower pregnancy rate compared to those patients with lower than 35.5% TUNEL-positive sperm (145). The conclusion to incorporate a new test into clinical practice depends on the volume and quality of reports that favor or refute such claims. Though multiple studies have analyzed the relationship between the degree of DNA damage and the fertilization rate, embryo cleavage rate, implantation rate, pregnancy rate, and live birth rate of offspring, accessible data on the correlation between abnormal DNA integrity and reproductive outcomes are limited and not analyzed systematically (86). The Practice Committee of the American Society for Reproductive Medicine summarizes the present understanding of the impact of abnormal sperm DNA integrity on reproductive outcomes (121). This Committee concluded that current methods for evaluating sperm DNA integrity merely do not predict pregnancy rates achieved with intercourse, IUI, or IVF and ICSI. Before sperm DNA damage investigation is introduced consistently in clinical practice, studies with sufficient sample size must be conducted assessing outcomes and role of such tests in the administration of male infertility (86).

13. Spermatozoa intergrity

Infertility is a growing problem among couples trying to conceive; in the past the female partner was singled out as the primary reason for being unable to bear a child. Research now reveals that male infertility may contribute in up to two thirds of all couples who seek treatment for infertility. For many years a conventional semen analysis (concentration, motility, and morphology) was seen as sufficient to diagnose male infertility; however, scientific examination must now take into account 2 different kinds of DNA that have been proven to contribute to this diagnosis. Nuclear DNA (nDNA), contained in the head of the sperm, is responsible for packaging all of the paternal genetic information that will be needed for the fertilized egg. nDNA can be damaged or compromised via 4 interrelated courses: defective chromatin packaging, apoptosis, oxidative stress, and genetic lesions. Mitochondrial DNA (mtDNA) is located in the midpiece of the sperm; when coupled with the tail, it is responsible for mobilizing the sperm toward the egg for fertilization. Scientists are only beginning to comprehend the relationship and interaction between these distinct DNA molecules and how they both contribute to male infertility. As the worldwide community continues to expand, an emerging subpopulation of couples has begun experiencing a common problem in making their contribution to the populace. These couples are experiencing a major health crisis, commonly referred to as infertility. Infertility is classically defined as a state in which a couple desiring a child is unable to conceive following 12 months of unprotected intercourse (146). It affects approximately 15% of couples who seek clinical treatment to conceive a child, and recent studies show that the number of infertile couples in the general population is growing (91). In infertile couples, responsibility for the lack of conception is generally divided into thirds, with one third due to male factors, one third due to female factors, and the final third due to overlapping factors from both partners.

The cornerstone of the evaluation of the man remains semen analysis. Although it gives some quantitative and qualitative information about the sperm sample, recent insight into the molecular biology and genetics of the sperm cell have demonstrated that morphology and motility alone are not the only grounds upon which sperm should be evaluated. Commonly overlooked is the fact that sperm carry 2 different kinds of DNA. The nDNA, commonly called the genome, is located in the head of the sperm. The second DNA type is called the mtDNA and is responsible for delivering the sperm to the egg by providing ATP for cellular acceleration. Both types of DNA work toward the common goal of fertilization, but each is susceptible to a myriad of factors that could derail the fertilization process. Imperfections in both types of DNA contribute equally to the problem at hand. This article hopes to elucidate male factor infertility as contributed by both kinds of DNA. Origins of nDNA Damage nDNA in somatic cell nuclei is packaged into structures called nucleosomes. These structures consist of a protein core formed by an octamer of histones with 2 loops of wrapped DNA. The nucleosomes are then further coiled into regular helixes called solenoids, which increase the volume of the chromatin (118). Sperm nuclei, however, need to be packaged much differently and more compactly to assure proper delivery of the nDNA. There are believed to be 4 levels of organization for packaging spermatozoon nDNA (118,147). One level consists of anchoring the chromosomes to the nuclear annulus. In another, DNA loop domains are created as the DNA attaches itself to the newly added nuclear matrix. The arrangement of these loop domains ensures that the DNA can be delivered to the egg in a form that is both physically and chemically accessible to the growing embryo. Chromosome repositioning and organization within the matrix of the sperm head is another level. Condensation of nDNA into tiny, supercoiled dough- nuts called toroids by replacing the nuclear histones with structures called protamines completes the levels of chromosomal organization. Human sperm contain 2 types of protamines that are about half the size of typical histones; throughout evolution, they have increased the number of positively charged residues, allowing formation of a highly condensed complex with the negatively charged paternal genomic DNA. Also, the addition of cysteine residues allows the formation of disulfide bonds between adjacent protamine molecules, thereby strongly stabilizing the nucleo-protamine complex (148). Prior to this re-arrangement, recombination is essential for spermatogenesis to occur (149); as seen in studies using animal knockout models, decreased recombination is associated with diminished spermatogenesis. Many factors (both endogenous and exogenous) can influence this, contributing to male infertility. Scientists agree on 4 distinct methods, although there may be others, by which nDNA can be compromised or damaged: defective sperm chromatin packaging, apoptosis, oxidative stress, and genetic lesions (150,151). The effects of these damaging methods are often found to be interconnected with defective chromatin packaging (2,13).

Chromatin packaging refers to the highly complex and specific structure into which nDNA is folded to properly deliver the genetic information to the egg. Although defects can arise at any of the 4 levels of packaging, the most common problems arise during DNA loop domain formation and histone-protamine replacement. nDNA loop domains can be difficult to arrange without inducing endogenous nicks to the nDNA (87,152). It is thought that these nicks exist naturally and serve to relieve torsional stress. The presence of these nicks is greatest during transition from round to elongated spermatids in the testis and occurs before complete protamination within the sperm. Topoisomerase II is the enzyme that creates aligates the nicks within nDNA during this process (153). Any defect inthe enzyme itself will negatively affect the packaging of the genetic information and will contribute to male infertility. The enzyme may leave the nDNA fragmented with single- or double-stranded breaks; this may indicate an early apoptotic process in somatic cells and incomplete sperm maturation in the case of spermatozoa. Topoisomerase inhibitors have been proven to increase the levels of internal nDNA breaks by preventing their repair and increasing their susceptibility to damage (154). Also involved in sperm chromatin packaging is the replacement of histones with protamines. Protamines are major DNA-binding proteins essential for chromatin condensation (155). During epididymal transport, histones are replaced by transition proteins, only to be replaced by protamines (156); both intermolecular and intramolecular disulfide cross-linking among the cysteine-rich protamines compresses the DNA into one sixth the volume occupied by somatic cell nDNA (157,158). This high rate of cross-linking affords the sperm nDNA a measure of protection against exogenous assault and compensates for an impaired DNA-repair capacity. Human spermatozoa retain approximately 5-15% histones in their structure, and proper histone-to-protamine exchange is critical for normal spermiogenesis, as aberrations in this replacement process are associated with male infertility and poor outcomes (159), perhaps to allow access for oocyte repair mechanisms. Human sperm contain 2 different types of protamines, which are believed to be present in equal amounts in fertile men: P1 and P2 (160). Experiments have shown that the ratio of P1 to P2 is critical to male fertility (161,162), more specifically to the sperm's fertilization ability (163). In addition, recent testing has demonstrated that P2 precursors (pre-P2) are vital in maintaining the delicate P1:P2 ratio. Defect in pre-P2 mRNA translation appears to cause abnormal sperm morphogenesis, reduced sperm motility, and subsequent male infertility. Also low pre-P2:P2 ratios suggest a link between deficient protamine processing and decreased nDNA integrity (164). Apoptosis is the controlled disassembly of a cell from within (165); it is believed to have 2 roles during normal spermatogenesis (166). The first role is to limit the germ cell population to numbers that can be supported by the surrounding Sertoli cells. The second is for the depletion of abnormal spermatozoa. As seen in the prior section, abnormal spermatozoa can be produced via defective sperm chromatin packaging, among other ways. In somatic cells, cells that enter into an apoptotic pathway usually have several classical indicators, such as phosphatidylserine (PS) relocation, Fas expression, nDNA strand breaks, and caspase activity. PS relocation is perhaps the earliest indicator of apoptosis; normally located on the inner leaflet of the plasma membrane, PS migrates to the outer membrane once the apoptotic signal has been given (145). To assist control this signal, both pro- and anti- apoptotic proteins are present in the testis; they are members of the Bcl-2 family of proteins and provide a signaling pathway that is imperative to maintaining male germ cell homeostasis (167). Bcl-2 and Bcl-xL are both prosurvival proteins, while Bax is a pro-apoptotic protein. Disturbing the balance of these proteins from the Bcl-2 family has been demonstrated in mice to contribute to male infertility by disrupting normal apoptosis levels. Fas expression is another indicator that the apoptosis signal has been given. Fas is a type I cell surface protein, belonging to the tumor necrosis/nerve growth factor receptor family (145); it is induced by the binding of Fas ligand to the Fas receptor on the plasma outer membrane. Sertoli cells are known to express Fas ligand, demonstrating the fact that apoptosis is a commonly used mechanism to control the germ cell population at a level that can be supported by the Sertoli cells (168). Ligation of Fas ligand to the Fas receptor triggers activation of cytosolic aspartate-specific proteases, or simply caspases. Once caspase activation has taken place, a signal is transduced to synthesize caspase-activated deoxyribonuclease, which leads to degradation of sperm DNA by forming singleand double-stranded breaks within the nDNA and apoptosis (169). In infertile men, ejaculated spermatozoa often possess partially degraded nDNA, usually considered to be indicative of the apoptosis pathway; this ‘‘escaping’’ of the apoptosis signal is referred to as ‘‘abortive apoptosis’’ (157,170). The apoptotic pathway is an all or nothing response, meaning that once the signal has been given there is no reversing the process. Abnormalities in this pathway are often attributed to 1 of 2 possibilities: infertile men may not produce enough sperm to trigger Sertoli cell activation to produce Fas, or there may be a problem in activating the Fas-mediated apoptosis signal (118,171). It is believed that if the apoptotic cascade is initiated at the round spermatid phase when transcription is still

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active, this may be the origin of the nDNA breaks commonly seen in abortive apoptosis in ejaculated spermatozoa. However, nDNA breaks are known to be common during condensation of the genome. It is currently unclear whether these breaks are caused by an aborted apoptotic pathway or simply by incomplete chromatin packaging. Also, not all caspase activity has been shown to be indicative of the apoptotic signal. Recent work has demonstrated that there appears to be some caspase activity in human germ cells that is not associated with apoptosis and may indeed serve a viable function (172,173). Another well-known inducer of the apoptotic pathway is telomere shortening. Telomeres are capping structures at chromosome ends that protect against rearrangements, preventing ends from being recognized as nDNA breaks (151,174). They are usually composed of tandem TTAGGG sequence repeats that are bound to a complex array of proteins. Telomerase is a specialized reverse transcriptase that contains a catalytic subunit that synthesizes new telomeric repeats. In the absence of telomerase, telomeric sequences are lost after each round of replication, eventually creating a shifted sequence that could be recognized as an nDNA double-stranded break; this would then be recognized by a genomic surveillance mechanism that appears in the elongating spermatid (143,151). This recognition is another way to induce an apoptotic response, possibly contributing to the ''abortive apoptosis'' theory. Abortive apoptosis is a theory that still requires much scientific evidence to be considered valid. Because of naturally occurring processes within the spermatozoa that mimic somatic cell apoptosis, many believe that this theory requires additional evidence. Oxidative stress upon spermatozoa is induced by an increase in the amount of reactive oxygen species (ROS) that are present in the fluids filling the male genital tract (175). Sperm are particularly susceptible to oxidative stress due to the high content of unsaturated fatty acids in their membranes, as well as their limited stores of antioxidant enzymes (176,177). Their increased susceptibility is enhanced by defective chromatin packaging, causing further damage to the genome; individuals with varicoceles are particularly susceptible to this type of damage (55,73).

ROS are created by metabolizing ground-state oxygen into the superoxide anion and H_2O_2 (178). They play an important physiologic role, modulating gene and protein activities vital to sperm proliferation, differentiation, and function (55). ROS also promote tyrosine phosphorylation to support sperm capacitation. Fertile men control ROS generation through seminal antioxidants; the pathogenic effects of ROS are apparent only when they are produced in excess of the antioxidant capabilities. It is known that the main source of excess ROS generation in semen is leukocytes; genital tract infections are considered to be the most common cause. However, secondary contributors are known to play an important role as well when an infection is not present. The origin of these secondary contributors has yet to be pinpointed in human sperm, but there are many sources under investigation. Three possible sources of excess ROS generation are from within the human sperm itself. The first is through mitochondria. Some electrons are leak out directly and transferred to O_2 to generate reactive oxygen species (ROS) in the ETC. (179). This was proposed because of tests performed on rat spermatozoa indicating increased transl ocation of mitochondrial free radicals into the sperm genome. However, further investigation has demonstrated that mitochondrial blockers do not have the same effects on human spermatozoa (170). The second proposed source is through Leukocytes contain an NADPH-oxidase (NOX) that catalyses the production of O_2 by the oxidation of NAD(P)H in sperm. This theoretic oxidase would serve to transfer electrons from NAD(P)H to ground-state oxygen to create the superoxide anion. It is known that NAD(P)H in leukocytes helps to contribute to ROS production in rat spermatozoa, but it has yet to be demonstrated in humans (180,181). The third proposed intracellular source of ROS, there is a subset of ROS called reactive nitrogen species (RNS) production is through the generation of nitric oxide (NO) can damage sperm DNA, affect male reproduction through its effects on sperm and the hypothalamic-pituitary-gonadal (HPG) axis (182,183). NO is a free radical created from the oxidation of L-arginine by 3 isoforms of nitric oxide synthase (NOS). NOS activity has been shown to be associated with the acrosome reaction and capacitation of mouse sperm, thus influencing their fertilizing potential. In humans, decreased NO concentrations are known to increase sperm capacitation and zona pellucida binding. The exact mechanism of its influence has yet to be elucidated. Other proposed sources of ROS come from outside the sperm's immediate environment, usually from outside of the host's body. Various types of pesticides all seem to have a common effect; they include xenobiotic agents such as organophosphorous pesticides that disrupt the endocrine system. These agents possess estrogenic properties that may induce oxidative stress oxidative stress (ROS/RNS production) by male germ cells and lead to hormonal dysfunctions (177,184,185). Cigarette smoking is also known to increase ROS levels through increased leukocyte generation. Infertile smokers are known to harbor increased levels of spermatid oxidative stress compared with infertile nonsmokers. This increase is associated with increased seminal leukocytes (186,187). Finally, scrotal heat stress has been demonstrated in stallions to damage sperm chromatin structure, possibly by oxidative stressors (188). There is a study similar analyses were performed on humans regarding the use of laptop computers in respect to elevated scrotal temperature can cause sperm to be abnormal in quantity and quality. When the scrotum heats up, the sperm that is produced can take on a different shape that is not as effective at penetrating the egg, lowering the chances of becoming pregnant (189). These findings also recognized the elevated temperature of the scrotal environment as having a negative effect upon spermatogenesis, warranting further research.

Genetic Lesions Genetic lesions are another possible means of attack through which nDNA can influence male infertility; these lesions create insults or gaps within the genome and may yield effects ranging from minimal to catastrophic. They can be divided into 3 classes based on the type of impact they present (190). The first class consists of chromosomal aneuploidies and rearrangements in which batteries of genes on specific chromosomes have changes in expression dosages or changes in their normal genomic environments. The second class embodies submicroscopic deletions (microdeletions), in which deletions or rearrangements of multiple genes mapped in a molecular environment have changes in their expression patterns. The third class is made up of single gene defects in which expression of a single gene (or key element) is changed or lost, causing male infertility. These lesions can affect all of the human chromosomes, including any of the 300 genes estimated to be involved in male fertility. They can occur within introns as well as exons, making their impact difficult to predict. Paternal

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nDNA Effects Prior to analyzing the second type of DNA found in spermatozoa, it is important to establish that nDNA integrity, as it relates to embryo quality, is still an intense topic of discussion. Paternal effects upon the embryo have been classified as both ‘‘early’’ and ‘‘late.’’ Early paternal effects appear to be mediated by centrosome destruction or a deficiency in oocyteactivating factors within the spermatozoa, implicating faulty sperm chromatin packaging and nDNA damage (191). Early effects are observed before the major activation of embryonic genome expression, which begins at the 4-cell stage in humans. Late paternal effects may involve sperm aneuploidy, nDNA damage, or abnormal chromatin packaging, which can influence the orderly activation of paternal gene expression (192). It has been found that there is no correlation between sperm nDNA fragmentation and the early paternal effect; however, many assisted reproductive technology (ART) clinics perform embryo transfers on the third day after embryo retrieval, prior to the time when late paternal effects can be fully observed. Because of this fact, blastocyst transfer may be preferable, at the risk of having fewer eggs to transfer mtDNA. The mtDNA of a sperm is completely located in the sperm midpiece; it exists as a circular, double-stranded DNA molecule composed of 16569 base pairs.

The most important function of the sperm mitochondria is to manufacture ATP. The mitochondria itself is composed of 2 distinct membranes, an inner membrane and an outer membrane. The outer membrane is relatively permissive and allows the passage of large molecules through nonspecific porin channels; the inner membrane is much more discriminatory. The inner membrane is heavily invaginated and forms cristae; enzymes for the ETC are located on the inner membrane, and the particular nature of inner membrane transport helps to maintain the mitochondrial membrane potential, which drives the ETC (193,194). It is important to remember the differences between mtDNA and nDNA (195,196). mtDNA is not afforded the same protection or basic upkeep that nDNA is given. First, there is no protection from histones or DNA-binding proteins within mtDNA; also it lacks introns. Because of this, every mutation in mtDNA has the potential to damage the function of the cell. mtDNA also lacks a significant proofreading system and replicates much more rapidly than nDNA; this causes the mutation rate found in mtDNA to be 10 to 100 times more than that of nDNA. mtDNA Deletions Because of the increased rate of occurrence, mitochondrial deletions have been exclusively investigated; of particular interest has been the relationship to sperm motility and forward progression. Deletions in the mitochondrial genome would directly affect the sperm’s ability to synthesize ATP through the ETC. Direct correlations have been found involving mtDNA deletions and decreased sperm motility (196). There are 6 distinct respiratory chain complexes that are required for the ETC to function properly. Amongst them, all but complex II are encoded by the mitochondrial genome; complex II is encoded by the nuclear genome and imported to the inner membrane of the mitochondria (197). Mitochondria and mitochondrial DNA are essential to sperm motility and fertility. It controls growth, development and differentiation through oxidation energy supply. Alterations in these multienzyme complexes are considered direct indications of mtDNA deletions. Deletions have been found to fall into 2 categories: small and large scale. While some large-scale deletions may be one of the important causes of dysfunction and non-motile sperm (197,198), small-scale deletions, however, can be equally devastating. Deletions as small as 2 base pairs have been proven to insert a stop codon into the mtDNA sequence and truncate vital proteins to ETC function (199). It is important to note that no single deletion has been found to be indicative of poor sperm quality. mtDNA deletions have also been compared to the ages of individuals seeking infertility treatment. Epididymal and testicular mtDNA deletions have also been compared, suggesting that testicular sperm may be superior to epididymal sperm for use in ART (195); however, recent publications suggest the opposite (200,201). Lastly, comparisons have been drawn between the incidences of nDNA deletions in combination with mtDNA deletions. Although results have only come out of a single laboratory, strong correlations between the 2 types of deletions have been found (195,196). mtDNA Copy Number The number of mtDNA molecules in a single spermatozoon is known as its mtDNA copy number. mtDNA copy number is controlled by the down-regulation of nuclear-encoded mitochondrial transcription factor A (202). Laboratories have been trying to pinpoint the specific mtDNA copy number that is considered normal for fertile men, but have had little success; reported copy numbers for normal, fertile men range from 3.8 in 100% density layers (202) to 74.1 6 2.0 in healthy men (203) to 717 6 394 in motile spermatozoa (204). These discrepancies are usually attributed to the method of analysis used or the crosshybridization of mitochondrial pseudogenes found in the nDNA. All reports, however, appear to correlate on one important fact: progressive cells possess fewer mtDNA copy numbers than do non-progressive spermatozoa. Mitochondrial DNA and Apoptosis While nDNA rearrangements can be associated with any number of possible abnormalities causing male infertility; mtDNA injury is most often attributed to the apoptotic pathway. Mitochondrial membrane potential is a measurable factor that has been used to predict the spermatozoa’s risk for apoptosis (205). There is an ongoing debate over the cause and effect of apoptotic signaling in mitochondria. In other words, does the sperm mitochondria respiratory system contribute to the ROS environment, causing apoptosis, or does the increased ROS environment cause mitochondrial respiratory failure?

A protein known as t-tpis, located in the testis and involved in spermatogenesis (full function unknown), has been given special attention due to its involvement in a vital Tom complex within the mitochondria of spermatozoa. Tom complexes are translocators of the mitochondrial outer membrane. T-tpis is found to be expressed solely in the midpiece of spermatozoa, linking it to possible mitochondrial function. Further investigation has revealed that t-tpis is a protein member of the Tom complex assembled using Tom 22 and Tom 40 complexes; they are known to be required for cell viability and are localized on the cytosolic side of the mitochondrial outer membrane. A potential ‘‘knob and key-hole’’ model involving t-tpis expression has been proposed as a possible way of paternal mitochondrial recognition and elimination. Contrary evidence of exclusive maternal mitochondrial inheritance comes from abnormal embryos that failed to eliminate paternal mtDNA; however, these embryos frequently fail to develop past the blastocyst stage (167,193). In the rare event that paternal mtDNA is observed in adults (206,207), recombination events are often attributed to this phenomenon. Nonetheless, it is generally more accurate to consider artificial recombination (ie, errors in testing) before considering actual recombination events to have occurred. Treatment of sperm DNA is performed for better ART outcomes. Unfortunately, there is no treatment for mtDNA deficiencies; instead, scientists have focused upon ways in which to isolate sperm with improved nDNA status, as well as selecting better sperm for ART use to generate better ART outcomes. However, recent evidence suggests the exact opposite, indicating epididymal sperm to be superior to testicular sperm for ICSI outcome (208,209). Also, a high-magnification optical system can be used to select better spermatozoa for ICSI. In this way, spermatozoa can be selected by visualizing morphology under conditions not possible with normal laboratory equipment. Subtle morphologic abnormalities become visible under this high magnification that cannot be seen under normal high-power objectives, allowing the embryologist to select better sperm for ICSI fertilization (210). Other ways to improve sperm nDNA include enhanced preparation techniques. This involves lowering the centrifugal forces exerted on the sperm when concentrating it and removing leukocytes as quickly as possible from the sample. Also the swim-up technique can be used to avoid use of the centrifuge. It is postulated that the addition of sperm wash medium to raw semen prior to liquefaction may inhibit bacterial binding to the sperm surface as well as diminish nDNA damage caused by ROS (118). Oddly, in vitro culture of surgically retrieved testicular spermatozoa for 48 to 72 hours at 37ºC has been suggested to enhanced motility but significant at 25 °C in azoospermia, along with decreasing the proportion of spermatozoa containing single-stranded nDNA breaks (211,212). A novel sperm selection assay was proposed to select viable sperm free of chromosomal anomalies for use with ICSI (213). Sperm hyaluronic acid (HA) binding has demonstrated the ability to isolate mature, viable sperm with unreacted acrosomal status, without damaging the specimen. One principle of this assay lies in the expression of the chaperone protein HspA2; in spite of its key role in meiosis, HspA2 levels have become indicative of sperm maturation (214). Low levels of HspA2 expression are associated with diminished sperm maturity, increased frequency of chromosomal aneuploidies, presence of apoptotic processes, and fragmented nDNA.

The second principle involved takes into sperm plasma membrane remodeling, cytoplasmic extrusion, and nuclear histone-protamine replacement, facilitating the formation of sperm binding sites for the zona pellucida of oocytes and for the binding sites of HA and has completed the spermiogenetic processes. Immature sperm that fail to undergo membrane remodeling are unable to bind to immobilized HA and will not be selected in this assay (213,214). Chromosomal disomies are said to be reduced between fourfold and fivefold in HA-selected sperm compared with semen sperm (215,216), indicating that HA preferentially selects for chromosomally normal sperm. Because of such promising results, a kit for this assay has become commercially available. The sperm-hyaluronan binding assay (HBA) has been marketed for routine testing of sperm motility and fertility (217). Unfortunately, HBA results have fallen well short of expectations in predicting successful fertilization rates in IVF, demonstrating less significance than sperm morphology and limiting its clinical predictive value.

14. Novel foods and food components versus spermatozoa intergrity

Diet is also directly associated with the integrity of spermatozoa and play vital roles to maintaining the proper functions. There are reports that food groups like dark green, leafy vegetables, fruits rich in vitamin C, walnuts rich in omega-3 fatty acids and arginine and whole cereals, antioxidants, liver, fatty fish, seafood, low-fat dairy products, coenzyme Q10 (CoQ10) and oysters contain the high level of zinc, vitamin B12, vitamin D, and Selenium have been positively play crucial roles in quality of sperm (218-221; Table 1). According to the available research studies suggest that consuming certain foods may harm the integrity of sperm such as high fat dietary products, trans fats, processed meat, soy products**,** coffee**,** sweet drinks, high level of carbohydrates are concern with structure and function of sperm cells (222,223; Table 1). High intakes of trans-fat, saturated fatty acids and other dietary components are related to higher oxidative stress that triggers inflammation via nuclear factor-kappa B (NF-kB) mediated cell signaling pathway together with the decrease in antioxidant activity which constitutes the underlying cause of decreased sperm quality and a higher risk of infertility due to deterioration of sperm morphology, as well as hormonal and immunological disorders (55,224). High-fat dairy products (whole milk, cream and cheese) were associated with decreased sperm motility and abnormal sperm shape. Some of this could be due to sex steroids given to cows (225). Increasing the level of ROS can lead to an increase in lipid peroxidation in sperm membrane, decrease in their flexibility, and ultimately a decrease in sperm motility. Also, oxidative stress may impair sperm axonemal and mitochondrial function, as well as DNA integrity, RNA and protein synthesis (55,226). As a result, change of lifestyle, mostly with regard to the antioxidant rich diet, low calorie foods seems to be crucial with regard to integrity of spermatozoa associated with male infertility. However, in-silico studies-based work using certain definite techniques, reviewed recently (227), is obligatory to bring in suitable application of certain specific tools in view of upgrading the quality of spermatozoa likely to be selected for application in all ART techniques to advance reproductive outcomes, namely, sperm count, sperm volume, sperm viability and thus male fertility. Nonetheless, these may provide new insights into expansion of novel male contraceptives.

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Ethical approval

It is a review article, and thus was not mandatory to get approval from Ethical Committee of the Institute, although all relevant reports/studies included in this overview have been duly cited in running text.

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Table 1. Nutraceutical effect of foods components on spermatozoa and fertility

Novel food	Nutritional components	Roles of foods components on spermatozoa and fertility
Spinach	Folic acid	Folic acid plays a vital role in spermatogenesis, the development of sperm cell. Required for DNA to synthesize properly during DNA replication. It reduces the number of abnormal sperms from the semen. Also increasing the chances of successful penetration of the sperm into the egg. Antioxidant like Vitamin A, C, E and lycopeneetc with protective benefits such as preventing free radicals from damaging DNA, may help protect the testis from harmful free radicals hence improving the quality, quantity, motility and integrity of sperm. Researcher found that consuming 4 mg of lycopene daily (found in a medium-sized tomato) enhanced men's sperm counts by on average 22 million/ml and motility by 25% and morphology 10%.
Tomatoes	Lycopene, Vit. C	
Guava	Zn, Vit. C & E	
Avocados	Folic acid	
Blueberries	Antioxidants	
Pomegranates	Folate, Vit. C	
Kiwis	Antioxidants	
Bananas	Vit. A, B1 and C	Banana consisting Bromelain an enzyme prevents inflammation resulting improve sperm quality and count.
Orange	Vitamin C	
Goji Berries	Natural antioxidant	Oyster Mushroom supplementation decreasing the genetic alterations and sperm abnormalities and improving sperm integrity.
Oyster Mushroom	Fats, Protein, niacin, riboflavin, Vit.B5, vit. B6, thiamin, P, K, Cu, Fe, Mg, zn, Mn, Se	
Maca Roots	Fiber, amino acids, vitamins & minerals	Increased volume of semen, sperm counts, possess better motility and fertility.
Dark Chocolate	L-Arginine HC	Contribute to higher sperm counts and volume.
Walnuts	Omega-3 fatty acids, arginine, Folate, B6, Zinc, Antioxidants	Nuts are required for the synthesis of the sperm cell membrane. Omega-3 fatty acids also help increase the volume of sperm by promoting blood flow to the testicles. Arginine contributes to the increase in sperm count of walnuts help in eliminating toxins substances from the blood.
Beef	Zinc, Se, Vitamin B 12, and Carnitine	Zinc is one of the most abundant mineral present in beef. Systematic review and meta-analysis revealed that Zn and Acetyl L-Carnitine (ALC) plays a crucial role in the improvement in sperm count, motility, morphology of sperm, maturation of sperm and improve several aspects of male fertility.
Liver	Vitamin A, Vitamin B12, Folate	Omega 3FAs can help to improve sperm motility, Morphology and sperm count enhancer.
Fish-Salmon	ω-3 fatty acids	Vitamin D directly associated with hormone production and high Vit. D levels increase overall semen quality.
Oysters	Protein, zinc, Se, B12, Fe, Mn, Cu, proteins, omega-3 FA, Vit. D	
Honey	Vit. B amino acids, Fe, Ca and minerals	Vitamin B is an essential component for the testosterone production. Testosterone plays crucial role in maintaining sex drive and sperm count.

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