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Physicochemical and Molecular Aspects of Mammalian Spermatozoa: An Overview

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Abstract
This succession involves the fusion of an oocyte with a sperm, proceeding to generation of a single diploid cell, the zygote, from that a new entity organism originates. The explanation of the physicochemical and molecular mechanisms of fertilization has captivated relevant researchers for a number of years. This review covers this enthralling succession at physiological, biochemical and molecular level. Various molecules have been documented to play a pivotal role in each step of this appealing process concerning to the sperm magnetism from the oocyte, the sperm maturation, the sperm and oocyte combination and the two gamete pronuclei union tending to the development of zygote. The collection of data concomitant with significant hypotheses enclosed by this overview provides novel insights into awaring the causes of fertility issues consequent to fertilization related disorders using the platform projecting in-silico studies.
Keywords: *Fertilization, zygote, gamete fusion, sperm capacitation, cortical reaction*

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I. 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 (Nanda *et al.,* 2010 a,b; Mishra and Nanda, 2011; Okabe, 2014). 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 (Mishra and Nanda, 2011), 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 (Hiltz *et al*., 2016). Certain arising views on mammalian spermatozoa function and interaction with egg i.e. fertilization are overviewed 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 (Philips *et al*., 2010). 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 their new shape will allow them to be 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 (Song *et al*., 2011). The process of spermatogenesis is a very productive and efficient way of producing a large number of sperm capable of fertilization. Harton and Tempest (2012) has comprehended that the incidence of abnormal sperm is basically an error in the process of spermatogenesis. However, it has been suggested that 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.

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 (Mital *et al*., 2011). 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 (Mishra *et al*., 2003 a, b. c; Nanda *et al*., 2010 a, b). Maturation of sperm predominantly takes place while sperm move from caput to the corpus sections of the epididymis (Mishra *et al*., 2003 a, b).

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 (Cornwall, 2009). 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 (Said and Reed, 2015). 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 (Said and Reed, 2015). 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 (Mishra and Nanda, 2011; Olugbenga et al., 2011). 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 (Mishra and Nanda, 2011) and glutamine in the bovine (Mishra *et al*., 2003 a,b).

Cornwall (2009) described a high molecular weight protein (>200 kd), located in the caudal epididymal fluid of the rat epididymis. 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. In these cases, the best alternative source of viable, reproductively capable sperm are those stored in the cauda epididymidis. Research as shown

that cauda epididymal sperm, when used with AI can produce offspring in a multitude of species, for example, the eland antelope, dog, gaur and Spanish ibex. 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 (Agarwal and Said, 2005) 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. Agarwal and Said (2005) reported that epididymal sperm respire at a much slower rate than ejaculated sperm, and from this observation they concluded that epididymal sperm is more efficient when it comes to the oxidative generation of utilizable energy. 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 (Martinez-Pastor *et al.,*2002). Epididymal and ejaculate sperm have been reported to respond differently to caffeine when used as a sperm motility stimulant (Agarwal and Said, 2005). 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 (Agarwal and Said, 2005).

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 (Matas *et al*., 2010).

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 (Golshan and Rezazadeh, 2013).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, (Golshan and Rezazadeh, 2013) reported that when epididymal sperm was collected into prostatic fluid of the dog there were less sperm possessing a pre-freeze cytoplasmic droplet. 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.

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 (Martinez-Pastor *et al.,*2002). Erenpreiss *et al.* (2006) 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.

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 (Martinez-Pastor *et al.,*2002). This method is often used in smaller species where the epididymides are difficult to manipulate due to their small size (Martinez-Pastor *et al.,*2002). 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. The third method of epididymal sperm collection is the retrograde flush method, which has been reported in the stallion (Briggs, 2003), African buffalo (Huszar et al., 2006) and Red deer (Martinez-Pastor *et al.,*2002). 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 (Martinez-Pastor *et al.,* 2002). 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. It has also been stated that the flushing method was an acceptable method to use out in the field when collecting epididymal sperm from African buffalo. In contrast, (Cary *et al*., 2004) 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. Due to the lack ofdifference between the two methods, they preferred the flotation method because it is easy to prepare (Cary *et al.,* 2004).

5. CLINICAL DIAGNOSIS AND ASSESSMENT OF SEMINAL OXIDATIVE STRESS

Spinal Cord Injury

Studies conducted during last two decades report the detection of increased ROS levels in the semen of 25% to 40% of infertile men (Cocuzza et al., 2007; Mishra and Nanda, 2011) documented that in men with spinal cord injury, elevated seminal ROS levels are associated with poor sperm motility and morphology. These associations are independent of both ejaculation method and specimen type (Cocuzza et al., 2007).

Leukocytospermia

ROS in the human ejaculate originate mainly from seminal leukocytes. Leukocytospermia is characterized by abnormally high seminal leukocyte, polymorphonuclear neutrophils, and macrophages (Mishra and Nanda, 2011). Patients with accessory gland infection demonstrate both leukocytospermia and elevated ROS levels. In these patients, sperm function defects are resultant of abnormal lipid peroxidation, stimulated by the high ROS levels.

Genito-Urinary (GU) Tract Infection

During GU infection, the presence of leukocytes in semen has been associated with decreased sperm motility and fertilization capacity (Khan *et al*., 2012). However, El-Demiry *et al*. reported no association between standard seminal parameters and leukocyte concentration in human semen (Khan *et al*., 2012). This dilemma may be partially due to the different techniques used to determine leukocyte concentration in semen as well as the lack of agreement on the lower leukocyte concentration responsible for sperm damage. Infections located in the testis and epididymis produce ROS that are particularly harmful to spermatozoa due to its lack of a pro-oxidant defense system. Sperm function may also be indirectly affected by an infection stimulating 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 (Rodrigo *et al*., 2004). Such findings support the controversial prostatitis-infertility relationship debate. Multiple hypotheses discuss male genital tract infections and their relationship with ROS. Specifically, the leukocytes stimulate human spermatozoa to produce ROS. The mechanisms responsible for such stimulation are unknown, but may include the direct contact of sperm and leukocytes or may be regulated by leukocyte release of soluble products (Rodrigo *et al*., 2004).

Environmental Factors

An association between cigarette smoking and reduced seminal quality has been identified (Agarwal and Said, 2005). Harmful substances including alkaloids, nitrosamines, nicotine, cotinine and hydroxycotinine are present in cigarettes and produce free radicals (Tesarik *et al.,* 2004). In a prospective study, Saleh *et al.* compared infertile men who smoked cigarettes with nonsmoker infertile men (Saleh *et al.,* 2002). Smoking was associated with a significant increase (approximately 48%) in seminal leukocyte concentrations, a 107% ROS level increase, and a 10 point decrease in ROS-TAC score. It could be concluded that infertile men who smoke cigarettes present higher seminal OS levels than infertile nonsmokers, possibly due to significant increase in leukocyte concentration in their semen. Significantly higher levels of DNA strand breaks in men who smoke have also been identified. DNA strand breaks may be resultant from the presence of carcinogens and mutagens in cigarette smoke. In recent decades evidence suggestive of the harmful effects of occupational exposure

chemicals known as endocrine disruptors on the reproductive system has gradually accumulated (Tesarik *et al.,* 2004). Environmental pollution is a major source of ROS production and has been implicated in the pathogenesis of poor sperm quality (Briggs, 2003). Tollgate workers with continuous environmental pollutant exposure had inversely correlated 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 (Briggs, 2003). In addition, the increase of industrialization has resulted in an elevated deposition of highly toxic heavy metals into the atmosphere. Paternal exposure to heavy metals such as lead, arsenic and mercury is associated with decreased fertility and pregnancy delay according to recent studies (Saleh *et al.,* 2002). Oxidative stress is hypothesized to play an important role in the development and progression of adverse health effects due to such environmental exposure.

6. FREE RADICALS AND ASSISTED REPRODUCTIVE TECHNIQUES

Numerous conditions associated 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 specific treatment. However, advances in ART have helped in improving treatment of male factor infertility (Briggs, 2003). ICSI is the most common ART method, although it is associated with the quality after ejaculation is density gradient centrifugation, migrationsedimentation, glass wool filtration, and conventional swim-up (Henkel *et al.,* 2004). The first three preparation techniques are more effective in reducing levels of free radicals than the conventional swim-up technique (Henkel *et al.,* 2004). Also high ROS levels are associated with decreased pregnancy rate following IVF or ICSI and arrested embryo growth. Based on a recent metaanalysis, which included all of the available evidence from the literature, our group found that there is a significant correlation between ROS levels in spermatozoa and the fertilization rate after IVF (estimated overall correlation 0.374, 95% CI 0.520 to 0.205) (Agarwal and Said, 2005). Thus, measuring ROS levels in semen specimens before IVF may be useful in predicting IVF outcome and in counseling selected patients with male factor or idiopathic infertility.

7. LABORATORY EVALUATION OF OXIDATIVE STRESS IN INFERTILITY PRACTICE

ROS Measurement

For clinical purposes, it is essential to have a reliable and reproducible method of ROS measurement. Numerous methods are available to measure ROS levels in semen (Homa *et al*., 2015). Direct methods such as electron-spin resonance spectroscopy, also known as electron paramagnetic resonance, have been utilized mainly for research purposes since these are relatively expensive technologies that require fresh samples, and great technical expertise. This method is used to detect electromagnetic radiation being absorbed in the microwave region by paramagnetic species that are subjected to an external magnetic field. This technique is the only analytical approach that permits the direct detection of free radicals and reports on the magnetic properties of unpaired electrons and their molecular environment. However, short life span of ROS makes the application of these techniques difficult.

Indirect techniques, e.g., chemiluminescence method are commonly used for measuring ROS produced by spermatozoa (Agarwal and Said, 2005). This assay quantifies both intracellular and extracellular ROS depending on the probe used. Chemiluminescence determines the amount of ROS, not the level of the sperm-damaging ROS present at any given time. Also, it can differentiate 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: luminol and lucigenin. A luminol-mediated chemiluminescence signal in spermatozoa occurs when luminol oxidizes at the acrosomal level. Luminol reacts with a variety of ROS and allows both intracellular and extracellular ROS to be measured. Lucigenin, however, yields a chemiluminescence that is more specific for superoxide anions released extracellularly (Agarwal and Said, 2005).

The number of free radicals produced is counted as photons per minute. Presence of leukocytes as a confounding factor and the need of fresh semen samples with high sperm count ($>1x10^6$/ mL) are the limitations of this technique (Agarwal and Said, 2005). Also other multiple factors that affect chemiluminescence include the concentration of reactants, sample volume, reagent injection, temperature control, instrument sensitivity, and background luminescence. A diversity of luminometers is available to measure the light intensity resulting from the chemiluminescence reaction. Single/double tube luminometers are sensitive and inexpensive but can measure only one or two samples at a given time, which are suitable for small research laboratories. On the other hand multiple tube or plate luminometers are more expensive since they can measure multiple samples at the same time and are suitable for centers that are engaged in regular research work on chemiluminescence (Agarwal and Said, 2005).

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 conceivable that measurement of oxidative stress can be made either by assessment of ROS or total antioxidant capacity (TAC). The TAC is measured by enhanced chemiluminescence assay or colorimetric assay (Briggs, 2003). It has been documented that a ROS-TAC score for assessment of seminal oxidative stress that showed to be superior to ROS or TAC alone in discriminating fertile and infertile population (Briggs, 2003). This score minimizes the variability 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 %, which was significantly higher ($p \leq 0.0002$) compared to infertile patient (35.8 %). The probability of successful pregnancy is estimated at < 10% for values of ROS-TAC < 30, but increased as the ROS-TAC score increased.

Leukocyte Evaluation

Since lower leukocyte levels are sometimes associated with significant ROS levels in semen it is important to determine the exact source of ROS in semen because the clinical implications of infiltrating leukocytes are quite different from those of pathological conditions in which spermatozoa themselves are the source of ROS (Briggs, 2003; Agarwal and Said, 2005). Methods that are currently used for assessment 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) can be used for assessment of seminal oxidative stress, and the differential contribution of cells to ROS generation, and to determine the state of activation of seminal leukocytes. ROS levels measured by chemiluminescence assay are strongly correlated with the results of NBT staining. Also, the NBT reduction test is commonly available, easily performed, inexpensive and has high sensitivity (Briggs, 2003; Agarwal and Said, 2005).

Oxidative Stress Status (OSS)

Currently there is no consensus regards to the inclusion of ROS measurement as part of the routine clinical evaluation of male infertility mainly because there is a lack of standardization of ROS analytical methods, equipment, and range of normal levels of ROS in semen. Some investigators have defined the basal levels of reactive oxygen species in neat semen specimens of normal healthy donors (Briggs, 2003; Agarwal and Said, 2005). Measurement of ROS levels in neat semen after liquefaction in the presence of seminal antioxidant protection proved to be a better test to evaluate oxidative stress status. The ROS levels for fertile donors with normal genital examination and normal standard semen parameters were 1.5×10^4 cpm/20 million sperm/mL. Using this value as a cutoff, infertile men can be classified as either OS-positive (> 1.5×10^4 cpm/20 million sperm/mL) or OS-negative ($\leq 1.5 \times 10^4$ cpm/20 million sperm/mL), irrespective of their clinical diagnosis or results of standard semen analysis (Agarwal and Said, 2005). Assessing ROS directly in neat semen showed diagnostic and prognostic capabilities identical to those obtained from ROS-TAC score (Agarwal and Said, 2005). Earlier studies have shown that sperm washing procedures like multiple centrifugation, resuspension, and vortexing artificially elevate ROS levels (Agarwal and Said, 2004). The antioxidant activity of seminal plasma is removed during sperm washing steps, which also results in elevated ROS levels. Excessive washing and manipulation including duration of centrifugation was found to be more important than the force of centrifugation for ROS formation by human spermatozoa. Therefore procedures that minimize multiple centrifugation, resuspension, and vortexing should be used for the preparation of spermatozoa for ART (Agarwal and Said, 2005).

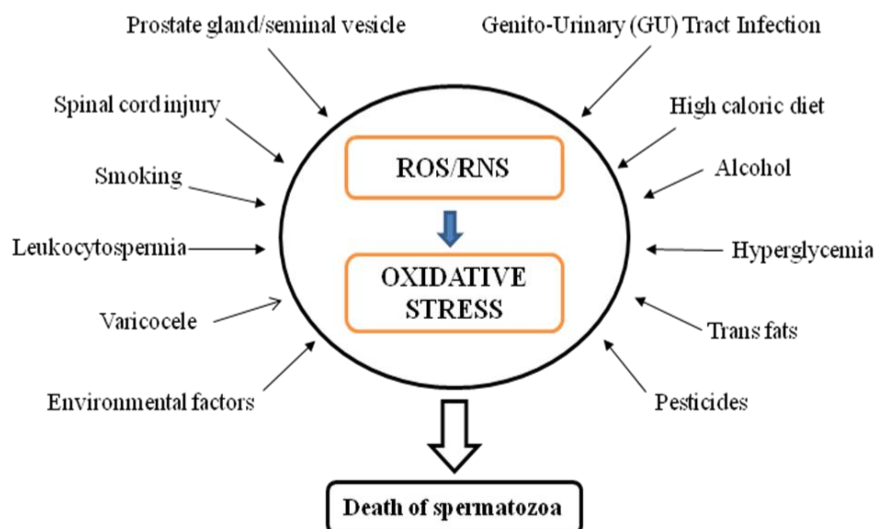

Figure 1: Sources of oxidative stress during spermatogenesis

Conflicting studies make it difficult to establish the clinical value of ROS measurement in medical practice since there is no clear evidence whether high ROS levels are a cause or an effect of abnormal semen parameters and sperm damage (Agarwal and Said, 2005). However, a more recent study reported high levels of ROS as an independent marker of male factor infertility, irrespective of whether these patients have normal or abnormal semen parameters (Agarwal and Said, 2005). Taken together the illustrations in Fig.1 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

Given the major role of oxidative stress in the pathogenesis of male infertility, treatment strategies with the goal of reducing levels of seminal oxidative stress are necessary for natural as well as assisted reproductive technologies. Spermatozoa produce small amounts of ROS that must be continuously inactivated to keep only the necessary amount to maintain normal physiologic cell function.

The pathologic levels of ROS detected in the semen of infertile men are more likely caused by increased ROS production than by reduced antioxidant capacity of the seminal plasma (Agarwal and Said, 2005; **Fig. 1**)). The body has a number of mechanisms to minimize free radical induced damage. Unfortunately, spermatozoa are unable to repair the damage induced by oxidative stress, because they lack the required cytoplasmic enzyme systems to perform the repair (Agarwal and Said, 2005). Antioxidants are the most important defense mechanisms against OS induced by free radicals. Metal chelators and metal binding proteins that block new ROS formation are classified as preventative antioxidants. Scavenger antioxidants, such as vitamins E and C, beta-carotene and other antioxidant dietary supplements, glutathione and enzymes, act via removing ROS already generated by cellular oxidation.

Many clinical trials have demonstrated the beneficial effect of antioxidants in treating selected cases of male infertility (Vernet *et al.*, 2001; Diez-Sanchez et al., 2003), whereas others failed to report the same benefits. Pregnancy, the most relevant outcome parameter of fertility, was reported in only a few of them (Vernet *et al.*, 2001; Diez-Sanchez et al., 2003). The majority of the studies analyze multiple antioxidant combinations, different dosages and durations. Also the patient‟s selection is another important aspect because oxidative stress can not be considered the cause of male infertility in all patients. Agarwal and Said (2005) in an extensive review of literature concluded that many studies suffer from the lack of placebo-controlled, double-blind design, making the effectiveness of antioxidant supplementation in infertile patients still inconclusive (Agarwal and Said, 2005).

Antioxidants may not be very effective depending on the etiology of infertility. Primarily, specific therapeutics directed against the etiological causes of elevated ROS should be attempted. Once the primary cause of infertility have been treated or no specific etiology is identified (idiopathic infertility) patients can be advised to take optimal doses of antioxidants supplementation.

9. ORIGIN OF DNA DAMAGE IN SPERMATOZOA

Sperm genetic material is structured in a special manner that keeps the nuclear chromatin highly stable and compact. The normal DNA structure is capable of decondensation at appropriate time transferring the packaged genetic information to the egg without defects in the fertilization process. The cause of DNA damage in sperm can be attributed to various pathological conditions including cancer, varicocele (Saleh *et al*., 2003), high prolonged fever (Saleh *et al*., 2003), advanced age (Saleh *et al*., 2003) or leukocytospermia (Erenpreiss *et al.,* 2006). Also a variety of environmental conditions can be involved as radiation (Saleh *et al.,* 2003), air pollution, smoking , pesticides, chemicals, heat and ART prep protocols (Erenpreiss *et al.,* 2006). Most of these agents not only disrupt hormone levels but may also induce oxidative stress, which could damage sperm DNA (Briggs, 2003). Higher sperm DNA damage in men exposure to pollutants. Nuclear DNA damage was inversely correlated with methaemoglobin levels. Infertile men with varicocele showed significant higher DNA damage that appears to be related to high OS. Normal semen has low DNA integrity and resist to leukocytospermia. Leukocytes increasing primary or provoking potential DNA damage. Significant increase in sperm DNA damage in leukocytospermic samples compared to normal controls. Higher sperm DNA damage in smokers compared to nonsmokers. Significant higher DNA damage in men > 36 years old. DNA damage is significantly higher in men over 40 years old Significant high DNA damage in men older than 35 years. Sperm DNA damage is higher in men with cancer even before cancer therapy. Recovery of spermatogenesis is higher when normal SCSA is found before adjuvant treatment. High sperm DNA damaged in these patients. Improvement in sperm chromatin packaging after chemotherapy. Detrimental effect on chromatin condensation and DNA integrity of cancer and as its treatment. 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 extent of sperm DNA damage has been closely coupled with impaired sperm function as well as male infertility. However 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 (Briggs, 2003). The most significant theories proposed as molecular mechanism of sperm DNA damage are: (a) defective chromatin packaging, (b) reactive oxygen species (ROS) (Saleh *et al*., 2003), (c) apoptosis principally during spermatogenesis (Saleh *et al*., 2003), and (d) DNA disintegration induced by endogenous endonucleases (Erenpreiss *et al.,* 2006).

10. ROLE OF OXIDATIVE STRESS IN SPERM DNA DAMAGE AS RELATED TO MALE INFERTILITY

Excessive generation of ROS in the reproductive tract not only affect the fluidity of the sperm plasma membrane, but also the integrity of DNA in the sperm nucleus. DNA bases are susceptible to oxidative damage resulting in base modification, strand breaks, and chromatin crosslinking. Oxidative stress-induced DNA damage causes pro-mutagenic change, which in its most severe form affects the quality of the germ line and prevents fertilization. When there is less oxidative damage, fertilization can occur, but the oocyte must repair the DNA strand breaks before the initiation of the first cleavage. Apoptosis and ROS are involved in mediating DNA damage in the germ line (Agarwal and Said, 2005). The Y chromosome is particularly vulnerable to DNA damage due to 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, whereas infertile men, in particular those with abnormal seminal parameters (**Fig. 2**), have higher fraction of sperm DNA damage. Idiopathic infertile men may present normal routine seminal parameters (concentration, motility, and morphology) with abnormal DNA integrity (Erenpreiss *et al.,* 2006; **Fig.2**).

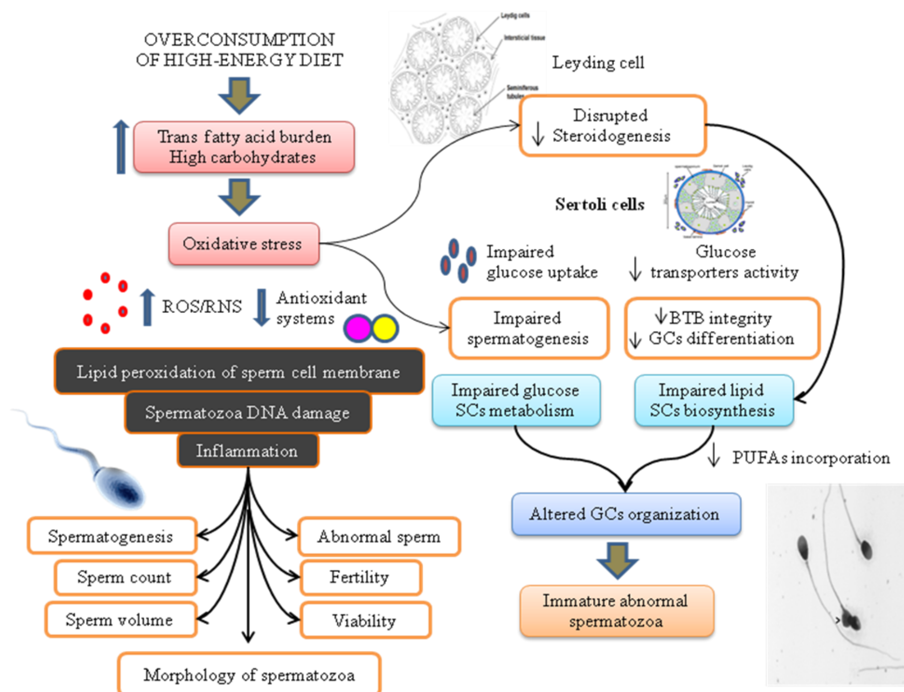

Figure 2: Impact of ROS/RNS on integrity of spermatozoa

It is of great concern that the most efficient ART techniques used to treat male factor infertility with high degree of sperm DNA damage. During ICSI, it is always desirable to select spermatozoa with normal morphology that reduces the risk of introducing spermatozoa with strand breaks (Vernet *et al.*, 2001). This is sometimes not always true since the traditional sperm parameters such as sperm count, motility and morphology have been proven to be poorly correlated to DNA damage status (Vernet *et al.*, 2001; **Fig.2**). Moreover, this has significant 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. These findings suggest that an estimate of the percentage of DNA damaged spermatozoa in fertile and infertile men may be important and a future challenge will be to develop methods to identify and select spermatozoa with intact DNA during the IVF/ ICSI procedures. Recently sperm from infertile men with varicoceles have been associated with significantly high levels of DNA damage (Saleh *et al.*, 2003). The finding of high seminal OS in patients with varicoceles may indicate that OS plays an important role in the pathogenesis of sperm DNA damage in patients with this condition. Although it has been reported that varicocelectomy can improve human sperm DNA integrity in infertile men with clinical varicoceles (Erenpreiss *et al.,* 2006); a limited number of studies have examined potential treatments to reduce sperm DNA damage. Therapeutic conditions have been suggested that avoidance of gonadotoxins; smoking, medications and hyperthermia (Vernet *et al.*, 2001); saunas and hot tubes may reduce sperm DNA damage. Treatment of GU infection can also be helpful based on the evidence that leukocytospermia induce ROS production and possibly DNA damage. Studies suggested that sperm DNA damage can be reduced with oral antioxidants administered during a relatively short time period. However, these recommendations have been based on small, uncontrolled studies and to date no treatment for abnormal DNA integrity has been shown to have successful clinical results (Lu *et al.,* 2018).

11. ASSESSMENT OF SPERM CHROMATIN INTEGRITY

Several techniques can measure DNA defects in human spermatozoa and the ability of these techniques to accurately estimate sperm DNA damage depends on many technical and biological aspects. However, to establish a threshold level between the fertile population and the lowest sperm DNA integrity required for achieving pregnancy remains extremely challenging. Currently both direct (fragmentation, oxidation) and indirect (sperm chromatin compaction) methods are available to evaluate 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 (Vernet *et al.*, 2001; Saleh *et al.,* 2003). Indirect methods mainly sperm chromatin integrity assays (SCSA) for assessing DNA damage uses chromatin and/or DNA intercalating dyes such as acridine orange to differentiate single-stranded and double-stranded DNA (Vernet *et al.*, 2001; Saleh *et al.,* 2003). Less frequent clinical tests for DNA damage include the sperm chromatin dispersion test (SCD) using the Halosperm kit, which allow to simultaneously perform DNA fragmentation and chromosomal analyses in the same sperm

cell (Erenpreiss *et al.,* 2006), liquid chromatography that detect oxidized DNA nucleotide residues (Vernet *et al.*, 2001) and evaluation of nuclear protein (protamine/histone ratio) levels in sperm samples.

All techniques currently 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 expressed as the DNA fragmentation index (DFI) (Lu *et al.*, 2018) that can distinguish 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 Oxidative Stress and Sperm Chromatin Damage in Male Infertility (Erenpreiss *et al.,* 2006). The main nuisance of ART is that they bypass the natural defense barrier present throughout female reproductive tract responsible for selecting the best spermatozoa for oocyte fertilization. Normally 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 (Saleh *et al.,* 2003). Standard semen parameters do not identify subtle defects in sperm chromatin architecture, which after the advent of ICSI has become more important parameter of sperm functional quality than count, motility or morphology. The emphasis on evaluation of genomic integrity has recently increased due to reports that correlate the degree of DNA damage with various fertility indices including rates of fertilization, embryo cleavage, implantation, pregnancy and live birth (Henkel *et al*., 2004).

Sperm DNA integrity is an essential requirement to achieve pregnancy in natural conception as well as for IVF outcomes where the natural process of fertilization is circumvented (Sheynkin *et al*., 2005). A high degree of sperm DNA damage has been found in couples presenting with unexplained recurrent pregnancy loss (Saleh *et al.,* 2003). All male partners of couples who achieved a pregnancy during the first 3 months attempting to conceive had < 30% sperm with fragmented DNA, whereas, 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. Moreover 84% of the men who initiated pregnancy before 3 months had sperm DNA damage levels of < 15%. Further, for IUI, there was a significantly higher chance of pregnancy/ delivery in the group with DFI < 27% and HDS (highly DNA stainable) of < 10% than in patients with DFI > 27% and HDS > 10%. Although, no statistical difference between the outcomes of IVF versus ICSI was observed in the group with DFI < 27%, ICSI had significantly better results than those of IVF in patients with DFI > 27%. The authors concluded that combining the two SCSA parameters, DFI and HDS is a useful method for prediction of IUI outcomes.

Henkel et al. (2004) reported that even though sperm DNA fragmentation did not correlate with the fertilization and embryo fragmentation rates, patients with a high percentage of TUNEL positive spermatozoa (> 36.5%) showed a significantly lower pregnancy rate compared to those patients with lower than 35.5% TUNEL-positive sperm. The decision to incorporate a new test into clinical practice depends on the volume and quality of reports that favor or refute such claims. Although 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, existing data on the relationship between abnormal DNA integrity and reproductive outcomes are limited and not analyzed systematically (Sheynkin *et al*., 2005). The Practice Committee of the American Society for Reproductive Medicine summarizes the current understanding of the impact of abnormal sperm DNA integrity on reproductive outcomes (Erenpreiss *et al.,* 2006). This Committee concluded that current methods for evaluating sperm DNA integrity alone do not predict pregnancy rates achieved with intercourse, IUI, or IVF and ICSI. Before sperm DNA damage analysis is introduced routinely in clinical practice, studies with adequate sample size must be conducted evaluating outcomes and role of such tests in the management of male infertility (Sheynkin *et al*., 2005).

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 (Vernet *et al.*, 2001). 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 (Saleh *et al.,* 2003). 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 (Agarwal and Said, 2005). 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 (Agarwal and Said, 2005). 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 (Erenpreiss *et al.,* 2006). Prior to this re arrangement, recombination is essential for spermatogenesis to occur (Erenpreiss *et al.,* 2006); 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 (Agarwal and Said, 2005; Lewis and Aitken, 2005; Shafik *et al*, 2006). The effects of these damaging methods are often found to be interconnected with defective chromatin packaging (Nanda *et al*., 2010 a, b; Mishra and Nanda, 2011).

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 (Vernet *et al.*, 2001). 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 an ligates the nicks within nDNA during this process (Henkel *et al.*, 2004). 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 (Lewis and Aitken, 2005). Also involved in sperm chromatin packaging is the replacement of histones with protamines. Protamines are major DNA-binding proteins essential for chromatin condensation (Henkel *et al.*, 2004). During epididymal transport, histones are replaced by transition proteins, only to be replaced by protamines (Shafik *et al*, 2006); 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 (Lewis and Aitken, 2005; Shafik *et al*, 2006). 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 15% histones in their structure, leading to a less compact chromatin arrangement than in other mammals (Erenpreiss *et al.*, 2006), 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 (Cocuzza *et al.*, 2007). Experiments have shown that the ratio of P1 to P2 is critical to male fertility (Henkel *et al.*, 2004; Erenpreiss *et al.,* 2006; Shafik *et al*, 2006; Carrell *et al.,* 2007), more specifically to the sperm‟s fertilization ability. In addition, recent testing has demonstrated that P2 precursors (pre-P2) are vital in maintaining the delicate P1:P2 ratio. Translation of pre-P2 mRNA appears to cause abnormal head morphogenesis, reduced sperm motility, and male infertility (Cocuzza *et al.*, 2007). Apoptosis is the controlled disassembly of a cell from within (Henkel *et al.*, 2004); it is believed to have 2 roles during normal spermatogenesis. 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 (Henkel *et al.*, 2004). 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 (Lewis and Aitken, 2005). 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 (Henkel *et al.*, 2004); 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 (Erenpreiss *et al.,* 2006). 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 DNA degradation by forming singleand double-stranded breaks within the nDNA (Agarwal and Said, 2005). 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"" (Lewis and Aitken, 2005; Erenpreiss *et al*., 2006). 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 Fasmediated apoptosis signal (Agarwal and Said, 2005). It is believed that if the apoptotic cascade is initiated at the round spermatid phase when transcription is still 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 (Martinez *et al.,* 2002). 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 (Shafik *et al.,* 2006). 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 (Shafik *et al.*, 2006). 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 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 (Tesarik *et al*., 2006). 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 (Baker and Aitken, 2005). 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 (Saleh *et al.*, 2003).

ROS are created by metabolizing ground-state oxygen into the superoxide anion and H_2O_2 (Vernet *et al*., 2001). They play an important physiologic role, modulating gene and protein activities vital to sperm proliferation, differentiation, and function (Erenpreiss *et al.,* 2006). 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 leakage of electrons from the mitochondrial transport chain (Vernet *et al*., 2001). This was proposed because of tests performed on rat spermatozoa indicating increased translocation 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 (Erenpreiss *et al.,* 2006). The second proposed source is through NADPH oxidase in sperm. This theoretic oxidase would serve to transfer electrons from NAD(P)Hto 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 (Vernet *et al.,* 2001; Baker and Aitken *et al.,* 2005). The third proposed intracellular source of ROS production is through the generation of nitric oxide (NO) (Balercia *et al.,* 2004). 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. They include xenobiotic agents such as organophosphorous pesticides that disrupt the endocrine system. These agents possess estrogenic properties that are capable of inducing ROS production by male germ cells (Baker and Aitken, 2005). 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 (Baker and Aitken, 2005). Finally, scrotal heat stress has been demonstrated in stallions to damage sperm chromatin structure, possibly by oxidative stressors. Recently similar analyses were performed on humans regarding the use of laptop computers in respect to elevated scrotal temperature (Sheynkin *et al.,* 2005). 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 (Vogt *et al*., 2004). 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 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 (Tesarik *et al*., 2002). 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 (Tesarik *et al.,* 2004). 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 16 569 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 transit 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 (St John *et al*., 2000 a,b). It is important to remember the differences between mtDNA and nDNA (O"Connell *et al*., 2002a). 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 (O"Connell *et al.,* 2002b). 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 (O"Connell *et al*., 2003). Dysfunctions in these 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 appear to be found in fertile men and may be considered „„common,"" they are usually associated with spermatozoa with low motility (St John *et al.,* 2000b; St John *et al*., 2001; O"Connell *et al*., 2002b). 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 (Thangaraj *et al.,* 2003). It is important to note that no single deletion has been found to be indicative of poor sperm quality (St John *et al*., 2001). 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 (O"Connell *et al.,* 2002a); however, recent publications suggest the opposite (Rodrigo *et al.,* 2004; Buffat *et al.,* 2006). 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 (O"Connell *et al*., 2002a; O"Connell *et al*., 2002b). 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 (May-Panloup *et al*., 2003). 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 (May- Panloup *et al.,* 2003) to 74.1 6 2.0 in healthy men (Kao *et al.,* 2004) to 717 6 394 in motile spermatozoa (Diez- Sanchez *et al.,* 2003). 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 1 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 (Marchetti *et al.,* 2002). 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 (St John *et al.,* 2000a). In the rare event that paternal mtDNA is observed in adults (Quintana-Murci *et al.,* 2001; Schwartz and Vissing, 2002; Bandelt *et al*., 2005), 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 (Rodrigo *et al.*, 2004; Buffat *et al.*, 2006). 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 (66006) that cannot be seen under normal high power objectives, allowing the embryologist to select better sperm for ICSI fertilization (Tesarik and Mendoza*,* 2007). 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 (Agarwal and Said, 2004). Oddly, in vitro culture of surgically retrieved testicular spermatozoa for 48 to 72 hours at 37uC has been suggested to improve motility, along with decreasing the proportion of spermatozoa containing single-stranded nDNA breaks (Emiliani *et al.,* 2001). Huszar *et al.* (2003) proposed a novel sperm selection assay to select viable sperm free of chromosomal anomalies for use with ICSI. 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 (Jakab *et al*., 2005). 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 account remodeling of the cytoplasmic and membrane-specific biochemical markers, facilitating the formation of sperm binding sites for the zona pellucida of oocytes and for the binding sites of HA. Immature sperm that fail to undergo membrane remodeling are unable to bind to immobilized HA and will not be selected in this assay (Jakab *et al.,* 2005). Chromosomal disomies are said to be reduced between fourfold and fivefold in HA-selected sperm compared with semen sperm (Huszar *et al.,* 2006), 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 (Ye *et al.,* 2006). 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, coenzymeQ10 (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 (Skoracka *et al*., 2020; Mishra *et al.,* 2021; Luna-Castillo *et al*., 2022; Ferramosca and Zara, 2022; **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 (Giahi *et al*., 2016; Ricci *et al*., 2017; **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-*k*B) 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 (Alahmar, 2019; Oteng AB, Kersten, 2020). 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 (Eslamian et al., 2012). 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 (Alahmar, 2019; Juan et al., 2021). 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.

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

Novel food	Nutritional components	Roles of foods components on spermatozoa and fertility
Spinach Tomatoes Guava Avocados Blueberries Pomegranates Kiwis Bananas Orange Goji Berries Oyster Mushroom	Folic acid Lycopene, Vit. C Zn, Vit. C & E Folic acid Antioxidants Folate, Vit. C Antioxidants Vit. A, B1 and C Vitamin C Natural antioxidant Fats, Protein, niacin, riboflavin, Vit.B5, vit. B6, thiamin, P, K, Cu, Fe, Mg, zn, Mn, Se	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%. Banana consisting Bromelain an enzyme prevents inflammation resulting improve sperm quality and count. Oyster Mushroom supplementation decreasing the genetic alterations and sperm abnormalities and improving sperm integrity.
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 Liver Fish-Salmon Oysters	Zinc, Se, Vitamin B 12, and Carnitine Vitamin A, Vitamin B12, Folate ω-3 fatty acids Protein, zinc, Se, B12, Fe, Mn, Cu, proteins, omega-3 FA, Vit. D	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. Omega 3FAs can help to improve sperm motility, Morphology and sperm count enhancer. Vitamin D directly associated with hormone production and high Vit. D levels increase overall semen quality.
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.

However, *in-silico* studies based work using certain definite techniques, reviewed by Saxena et al. (2013), 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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References

[1]. Agarwal, A. and Said, T.M. (2005). Oxidative stress, DNA damage and apoptosis in male infertility: A clinical approach. Br. J. Urol. Int., 95: 503-507.
[2]. Alahmar, A.T. (2019). Role of Oxidative Stress in Male Infertility: An Updated Review. J Hum Reprod Sci 2019; 12(1): 4-18.
[3]. Baker, M.A. and Aitken, R.J. (2005). Reactive oxygen species in spermatozoa: Methods for monitoring and significance for the origins of genetic disease and infertility. Reprod. Biol. Endocrinol., 3: 67-75.

[4]. Balercia, G., Moretti, S., Vignini, A. , Magagnini, M. and F. Mantero, F. *et al*. (2004). Role of nitric oxide concentrations on human sperm motility. J. Androl. 25: 245-249.

[5]. Bandelt, H.J., Q.P. Kong,O.P., Parso, W. and Salas, A. (2005). More evidence for nonmaternal inheritance of mitochondrial DNA? J. Med. Genet., 42: 957-960.

[6]. Briggs, D. (2003). Environmental pollution and the global burden of disease. British Medical Bulletin 68 (1): 1–24.

[7]. Buffat, C., Patrat, C., Merlet, F., Guibert, J. and S. Epelboin, S. *et al*. (2006). ICSI outcomes in obstructive azoospermia: Influence of the origin of surgically retrieved spermatozoa and the cause of obstruction. Hum. Reprod. 21: 1018-1024.

[8]. Cary, J. A., Madill, S., Farnsworth, K., Hayna, J. T., Duoos, L. and Fahning, M. L. (2004). A comparison of electroejaculation and epididymal sperm collection techniques in stallions. The Canadian Veterinary Journal 45(1): 35–41.

[9]. Cocuzza, M., Sikka, S. C., Athayde, K. S. and Agarwal, A. (2007). Clinical relevance of oxidative stress and sperm chromatin damage in male infertility: an evidence-based analysis. International Braz. J. Urol. 33(5): 603–621.

[10]. Cornwall, G.A. (2009). New insights into epididymal biology and function. Hum. Reprod. Update 15 (2): 213–227.

[11]. Diez-Sanchez, C., Ruiz-Pesini, E., Lapena, A.C., Montoya, J., Perez-Martos, A., Enriquez, J.A. and Lopez-Perez, M.J. (2003). Mitochondrial DNA content of human spermatozoa. Biol. Reprod. 68: 180-185.

[12]. Emiliani, S., Van den Bergh, M., Vannin, A.S., Biramane, J., Verdoot, M. and Englert, Y. (2001). Evidence of reduced single-stranded testicular sperm DNA from obstructive azoospermic men after 3 days of *in-vitro* culture. Hum. Reprod. 16: 1200-1203.

[13]. Erenpreiss, J., Bungum, M., Spano, M., Elzanaty, S., Orbidans, J. and Giwercman, A. (2006). Intra-individual variation in sperm chromatin structure assay parameters in men from infertile couples: clinical implications. Hum. Reprod. 21:2061–2064.

[14]. Eslamian, G., Amirjannati, N., Rashidkhan, B., Sadeghi, M.R. and Hekmatdoost, A. (2012). Intake of food groups and idiopathic asthenozoospermia: a case-control study. Hum. Reprod. 27: 3328-3336.

[15]. Ferramosca, A. and Zara, V. (2022). Diet and Male Fertility: The Impact of Nutrients and Antioxidants on Sperm Energetic Metabolism. Int. J. Mol. Sci. 23(5): 2542-2546.

[16]. Fode, M., Krogh-Jespersen, S., Brackett, N.L., Dana, A., Ohl, D.A., Lynne, C.M. and Sønksen, J. (2012). Male sexual dysfunction and infertility associated with neurological disorders. Asian Journal of Andrology 14 (1): 61-68.

[17]. Giahi, L., Mohammadmoradi, S., Javidan, A. and Sadeghi, M.R. (2016). Nutritional modifications in male infertility: a systematic review covering 2 decades. Nutr Rev ; 74(2): 118-130.

[18]. Golshan, I. F. and Rezazadeh, V. M. (2013). The epididymal sperm viability, motility and DNA integrity in dead mice maintained at 4-6oC. Iranian Journal of Reproductive Medicine, 11(3): 195–200.

[19]. Harton, G.L. and Tempest, H.G. (2012). Chromosomal disorders and male infertility. Asian Journal of Andrology 14 (1): 32-39.

[20]. Henkel, R., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., Menkveld, R., *et al*. (2004). Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril: 81: 965–972.

[21]. Hilz, S., Andrew J., Modzelewski, P.E. and Cohen, A.G. (2016). The roles of microRNAs and siRNAs in mammalian spermatogenesis. Development 143 (17): 3061-3073.

[22]. Homa, S. T., Vessey, W., Perez-Miranda, A., Riyait, T. and Agarwal, A. (2015). Reactive Oxygen Species (ROS) in human semen: determination of a reference range. Journal of Assisted Reproduction and Genetics, 32(5): 757–764.

[23]. Huszar, G., C. Celik-Ozenci, S. Cayli, Z. Zavaczki, E. Hansch and L. Vigue, 2003. Hyaluronic acid binding by human sperm indicates cellular maturity, viability and unreacted acrosomal status. Fertility Sterility 9: 1616-1624.

[24]. Huszar, G., Ozkavukcu, S., Jakab, A., Celik-Ozenci, C., Sati, G.L. and Cayli, S. (2006). Hyaluronic acid binding ability of human sperm reflects cellular maturity and fertilizing potential: Selection of sperm for intracytoplasmic sperm injection. Curr. Opin. Obstetrics Gynecol. 18: 260-267.

[25]. Jakab, A., Sakkas, D., Delpiano, E., Cayli, S. and Kovanci, E. *et al*. (2005). Intracytoplasmic sperm injection: A novel selection method for sperm with normal frequency of chromosomal aneuploidies. Fertility Sterility 84: 1665-1673.

[26]. Juan, C.A., Jose, de la, Lastra, M.P., Plou, F.J. and Perez-Lebena, E. (2021).The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. Int. J. Mol. Sci. 22(9): 4642-4646.

[27]. Kao, S.H., Chao, H.T., Liu, H.W., Liao, T.L. and Wei,Y.H. (2004). Sperm mitochondrial DNA depletion in men with asthenospermia. Fertility Sterility 82: 66-73.

[28]. Khan, M. S., Mohammad, S. H., Deepa, F., and Tahir, F. (2012). Association between Pus Cells and Semen Parameters in Infertile Pakistani Males. Sultan Qaboos University Medical Journal, 12(4): 479–484.

[29]. Lewis, S.E.M and R.J. Aitken, 2005. DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res.: 322: 33-41.

[30]. Lu, J.-C., Jing, J., Chen, L., Ge, Y.-F., Feng, R.-X., Liang, Y.-J., and Yao, B. (2018). Analysis of human sperm DNA fragmentation index (DFI) related factors: a report of 1010 subfertile men in China. Reproductive Biology and Endocrinology 16 (1): 2-9.

[31]. Luna-Castillo, K.P., Olivares-Ochoa, X.C., Hernandez-Ruiz, R.G., Llamas-Covarrubias, I.M., Rodriguez-Reyes, S.C., Betancourt-Nunez, A., Vizmanos, B., Martínez-Lopez, E., Munoz-Valle, J.F., Marquez-Sandoval, F., Lopez-Quintero, A. (2022). The Effect of Dietary Interventions on Hypertriglyceridemia: From Public Health to Molecular Nutrition Evidence. Nutrients; 14(5): 1104-1107.

[32]. Marchetti, C., Obert, G., Deffosez, A., Formstecher, P. and Marchetti, P. (2002). Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Hum. Reprod., 17: 1257-1265.

[33]. Martinez, F., Rienzi, L., Iacobelli, M., Ubaldi, F. and Mendoza, C. *et al*. (2002). Caspase activity in pre-implantation human embryos is not associated with apoptosis. Hum. Reprod. 17: 1584-1590.

[34]. May-Panloup, P., Chretien, M.F., Savagner, F., Vasseur, C. and Jean, M. *et al*., 2003. Increased sperm mitochondrial DNA content in male infertility. Hum. Reprod. 18: 550-556.

[35]. Matás, C., Sansegundo, M., Ruiz, S., García-Vázquez, F. A., Gadea, J., Romar, R., and Coy, P. (2010). Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa. Theriogenology 74(8): 1327–1340.

[36]. Mishra, S., Chauhan, S.K. and Nayak, P. (2021). Physiological, biochemical, biotechnological and food technological applications of Mushroom: An overview. IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) 7 (1): 39-46.

[37]. Mishra, S., Somnath, P.R. and Vijayaraghavan, S. (2003a). Distinct pools of PP1 gamma2 are regulated by sds22, protein 14-3-3 and hsp90 within spermatozoa. Experimental Biology & Translating the Genome Meeting, April 11-15: San Diego, CA, USA: Abstract number: LB270.

[38]. Mishra, S., Somnath, P.R., Huang, Z. and Vijayaraghavan, S. (2003b). Binding and Inactivation of the Germ Cell-Specific Protein Phosphatase PP172 by sds22 During Epididymal Sperm Maturation. Biology of Reproduction 69: 1572- 1579.

[39]. Mishra, S., Huang, Z. and Vijayaraghwan, S. (2003c). Binding to sds22 and inactivation of catalytic activity of the protein phosphatase PP1 gamma2 occurs dutring sperm motility initiation in epididymis. 36th Annual Meeting of Society for the Study of Reproduction, July 19-22: Cincinati, Ohio, USA.

[40]. Mishra, S. and Nanda, S. (2011). Sperm DNA bridges from sperm to egg to inculcate genetic variability: A review. Asian Journal of Biotechnology 3: 22-37.

[41]. Mital, P., Hinton, B.T. and Duffour, J.M. (2011). The blood-testis and blood-epididymis barriers are more than just their tight junctions. Biology of Reproduction 84 (5): 851-858.

[42]. Nanda, S., Mishra, S., Varshney, V.P. and Singh, R.B.(2010a). A biotechnological approach to apoptosis of somatic and germ cells in living organisms. Open Nutraceuticals J. 3: 81-93.

[43]. Nanda, S., Varshney, V.P. and Mishra, S. (2010b). Biochemical characterization of the mechanism of binding of a regulatory protein to the spermatozoa- specific protein phosphatase in *Swiss albino* rat. J. Applied Biosci. 27: 1715-1726.

[44]. O"Connell, M., McClure, N. and Lewis, S.E.M. (2002a). Mitochondrial DNA deletions and nuclear DNA fragmentation in testicular and epididymal human sperm. Hum. Reprod. 17: 1565-1570.

[45]. O"Connell, M., N. McClure, N. and S.E.M. Lewis, S.E.M. (2002b). A comparison of mitochondrial and nuclear DNA status in testicular sperm from fertile men and those with obstructive azoospermia. Hum. Reprod. 17: 1571-1577.

[46]. O"Connell, M., McClure, N., Powell, L.A., Steel, E.K. and Lewis, S.E.M. (2003). Differences in mitochondrial and nuclear DNA status of high density and low-density sperm fractions after density gradient preparation. Fertility Sterility 79: 754-762.

[47]. Okabe, M. (2014). Mechanism of fertilization: A modern view. Exp. Anim. 63 (4): 357-365.

[48]. Olugbenga, O. M., Olukole, S. G., Adeoye, A. T., and Adejoke, A. D. (2011). Semen characteristics and sperm morphological studies of the West African Dwarf Buck treated with Aloe vera gel extract. Iranian Journal of Reproductive Medicine 9(2): 83–88.

[49]. Oteng, A.B. and Kersten, S. (2020). Mechanisms of Action of *trans* Fatty Acids. Adv. Nutr. 11(3): 697-708.

[50]. Phillips, B.T., Gassei, K. and Orwig, K.E. (2010). Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond B Biol Sci. 365 (1546): 1663–1678.

[51]. Quintana-Murci, L., Rotig, A. Munnich, A., Rustin, P. and Bourgeron, T. (2001). Mitochondrial DNA inheritance in patients with deleted mtDNA. J. Med. Genet. 38: e28-e28.

[52]. Ricci, E., Vigano, P., Cipriani, S., Somigliana, E., Chiaffarino, F., Bulfoni, A. and Parazzini, F. (2017). Coffee and caffeine intake and male infertility: a systematic review. Nutr J. 16: 1-14.

[53]. Rodrigo, L., Rubio, C., Mateu, E., Simon, C., Remohi, J., Pellicer, A. and Gil-Salom, M. (2004). Analysis of chromosomal abnormalities in testicular and epididymal spermatozoa from azoospermic ICSI patients by fluorescence in-situ hybridization. Hum Reprod. 19: 118-123.

[54]. Said, A.-H. and Reed, M. L. (2015). Increased count, motility, and total motile sperm cells collected across three consecutive ejaculations within 24 h of oocyte retrieval: implications for management of men presenting with low numbers of motile sperm for assisted reproduction. Journal of Assisted Reproduction and Genetics 32(7): 1049–1055.

[55]. Saleh, R., Agarwal, A., Sharma, R., Said, T., Sikka, S. and Jr. Thomas, A. (2003). Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. Fertil. Steril. 80: 1431-1436.

[56]. Sanchez-Pena, L.C., Reyes, B.E., Lopez-Carrillo, L., R. Recio, R. and Moran-Martinez, J. *et al*., (2004). Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers. Toxicol. Applied Pharm. 196: 108-113.

[57]. Saxena, A., Sangwan, R.S. and Mishra, S. (2013). Fundamentals of homology modeling steps and comparison among important bioinformatics tools: An overview. Science International 1(7): 237-252.

[58]. Schwartz, M. and Vissing, J. (2002). Paternal inheritance of mitochondrial DNA. New Engl. J. Med. 347: 576-580.

[59]. Shafik, A., Shafik, A.A., Shafik, I. and Sibai, O. (2006). Sperm DNA fragmentation. Arch Androl 52: 197-208.

[60]. Sheynkin, Y., Jung, M., Yoo, P., Schulsinger, D. and Komaroff, E. (2005). Increase in scrotal temperature in laptop computer users. Hum. Reprod. 20: 452-455.

[61]. Skoracka, K., Eder, P., Lykowska-Szuber, L., Dobrowolska, A., Krela-Kazmierczak, (2020) I. Diet and Nutritional Factors in Male (In)fertility-Underestimated Factors. J. Clin. Med. 9(5): 1400-1402.

[62]. Song, N., Liu, J. (2011). An, S. Tomoya, N. Yoshitaka, H. and Takehiko, K. (2011). Immunohistochemical analysis of histone H3 modifications in germ cells during mouse spermatogenesis. Acta Histochemica et Cytochemica. 44 (4): 183–90.

[63]. St John, J., Sakkas, D., Dimitriadi, K., Barne, A. and Maclin, V. *et al*., 2000a. Failure of elimination of paternal mitochondrial DNA in abnormal embryos. Lancet 355: 200-200.

[64]. St John, J.C., Sakkas, D. and Barratt, C.L. (2000b). A role for mitochondrial DNA and sperm survival. J. Androl. 21: 189-199.

[65]. St John, J.C., Jokhi, R.P. and Barratt, C.L. (2001). Men with oligoasthenoteratozoospermia harbour higher numbers of multiple mitochondrial DNA deletions in their spermatozoa, but individual deletions are not indicative of overall aetology. Mol. Hum. Reprod., 7: 103-111.

[66]. Tesarik J, Mendoza-Tesarik R and Mendoza C. (2006). Sperm nuclear DNA damage: update on the mechanism, diagnosis and treatment. Reprod Biomed Online 12: 715-21.

[67]. Thangaraj, K., Joshi, M.B., Reddy, A.G., Rasalkar, A.A. and Singh, L. (2003). Sperm mitochondrial mutations as a cause of low sperm motility. J. Androl. 24: 388-392.

[68]. Tesarik, J. and Mendoza, C. (2007). Treatment of severe male infertility by micromanipulation-assisted fertilization: An update. Frontiers Biosci 12: 105-114.

[69]. Tesarik, J., Mendoza, C. and Greco, E. (2002). Paternal effects acting during the first cell cycle of human pre-implantation development after ICSI. Hum. Reprod. 17: 184-189.

[70]. Tesarik, J., Greco, E. and C. Mendoza, C. (2004). Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. Hum. Reprod. 19: 611-615.

[71]. Vernet, P., Fulton, N., Wallace, C. and R.J. Aitken, R.J. (2001). Analysis of reactive oxygen species generating systems in rat epididymal spermatozoa. Biol. Reprod., 65: 1102-1113.

[72]. Vogt, P. (2004.) Molecular genetics of human male infertility: From genes to new therapeutic perspectives. Curr. Pharm. Design 10: 471-500.

[73]. Ye, H., Huang, G., Gao, Y. Y. and Liu, D.Y. (2006). Relationship between human sperm hyaluronan binding assay and fertilization rate in conventional in vitro fertilization. Hum. Reprod. 21: 1545-1550.

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