

Sperm Chromatin Integrity: Etiologies and Mechanisms of Abnormality, Assays, Clinical Importance, Preventing and Repairing Damage

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Abstract

The standard semen analysis is the first line and the most popular laboratory test in the diagnosis of male fertility. It evaluates sperm concentration, motility, morphology and their vitality. However, it is well-known that normal results of semen analysis can not exclude men from the causes of couples' infertility. One of the most important parameters of sperm in its fertilizing potential is "Sperm chromatin integrity" that has direct positive correlation with Assisted Reproductive Techniques (ART) outcomes including; fertilization rate, embryo quality, pregnancy and successful delivery rate. It seems that sperm DNA chromatin integrity provides better diagnostic and prognostic approaches than standard semen parameters. For these reasons understanding the sperm chromatin structure, etiology of sperm chromatin abnormality, identification factors that disturbs sperm chromatin integrity and the mechanism of their action can help in recognizing the causes of couples' infertility. Various methods of its evaluation, its importance in male fertility, clinical relevance in the outcomes of ART and application of laboratory and medical protocols to improve this integrity have valuable position in diagnosis and treatment of male infertility. There has recently been interest in the subject and its application in the field of andrology. Therefore, with regard to the above mentioned importance of sperm chromatin integrity, this review article describes details of the useful information pertaining to sperm DNA damage including the origins, assessments, etiologies, clinical aspects, and prevention of it.

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Introduction

Infertility is a major problem in 15-20% of couples trying to conceive in the reproductive ages⁽¹⁾. Male infertility may contribute in half of all couples who refer to infertility clinics⁽²⁾.

The standard semen analysis is the first line and the most popular laboratory test in the diagnosis of male fertility. It evaluates sperm concentration, motility, morphology and vitality. However, it is well-known that normal results of semen analysis can not exclude men from causes of couples' infertility⁽³⁾.

Today, it is well known that the quality and integrity of sperm chromatin is very important in the reproductive potential of men. Sperm DNA is known to contribute to half of the genomic material of the embryo. Sperm chromatin is much more compact in somatic cells, with the aim of protecting the paternal genetic materials against damaging factors during its passage from testis as production site to fallopian tubes as final destination⁽⁴⁾.

It is a fact that normal sperm genomic

material is required for a normal fertilization, pregnancy, live birth, and postnatal child well being. Therefore, abnormal sperm chromatin may result in male sub-fertility or even infertility, recurrent abortion, increased congenital anomalies and testicular cancer in offsprings⁽⁵⁾. Thus, more knowledge on sperm chromatin damage including: etiology, mechanism, detection methods, clinical outcomes and treatment may provide better diagnostic and prognostic capabilities than standard sperm parameters for both *in vivo* and *in vitro* fertilizing ability of human spermatozoa⁽⁶⁾.

Etiology of sperm chromatin damage

Many factors are involved in inducing sperm DNA damage, which may result in the male infertility. One of these factors is Leukocytospermia⁽⁷⁾, which is the increase of leukocytes in semen due to genital infection and/or inflammation⁽⁸⁾.

Leukocytospermia could result in the production of inflammatory cytokines followed by the overproduction of Reactive Oxygen Species (ROS) leading to increase in sperm DNA damage^(9,10). Cigarette smokes also induce leukocytospermia and ROS overproduction⁽¹¹⁻¹³⁾. It seems that leukocytes can generate high levels of ROS in semen, which may overwhelm the antioxidant capacity of semen and resultant Oxidative Stress (OS) could disturb sperm functions^(14, 15).

Also, some of the sperm preparation and cryopreservation protocols could decrease sperm chromatin integrity^(16,17). It has been shown that rapid and ultra rapid freezing (flash-freezing in liquid nitrogen) induce the least damage to sperm during cryopreservation⁽¹⁸⁾. Malignancies such as leukemia, Hodgkin's disease, and testicular malignancies could affect sperm chromatin integrity by themselves or following their treatment with cytotoxic drugs and/ or radiation therapy⁽¹⁹⁻²²⁾.

Many drugs might also affect the semen quality and increase sperm DNA damage⁽²³⁻²⁵⁾. Even some types of herbal remedies may induce sperm DNA damage. For example high dose administration of Ginkgo

biloba, St. John's wort and Echinaceae purpura were associated with increased damage of the reproductive cells⁽²⁶⁾.

Finally, a growing body of evidences shows that environmental and occupational exposures to chemical agents, heat, and agricultural toxins could play a role in sperm DNA damage⁽¹⁰⁾. In a recent study, it has been shown that increased scrotal heat not only reduces the quality of semen parameters, but also compromises sperm chromatin integrity⁽²⁷⁾.

Mechanism of Sperm DNA Damage

While the exact causes of sperm DNA damage have not yet been fully elucidated, several interrelated mechanisms have been suggested⁽²⁸⁻³⁰⁾. These mechanisms are categorized as abnormal chromatin packaging, apoptosis and oxidative stress.

Abnormal chromatin packaging

The sperm chromatin is an extremely compact and stable structure which must be organized in a specific manner to achieve this unique condensed state⁽³¹⁾. This DNA organization provides more safe and secure transfer of paternal genetic information to the egg and next generation. Sperm chromatin is organized in a manner completely different from that of somatic cells^(32, 33).

During changes in sperm chromatin compaction, histones are replaced by transition proteins. These proteins are then replaced by more basic proteins named protamine (P1,P2), which are responsible for the final condensation and stabilization of sperm DNA⁽³⁴⁾. Sperm DNA interacts with protamines so that it converts the coiling of sperm DNA into toroidal subunits so called as "Doughnut loops"⁽³⁵⁾.

Although defects can arise at any stage of this process, the most common problems are due to abnormal DNA loop domain formation and histon-protamine replacement⁽³⁴⁾.

DNA loop domains are arranged by endogenous nicks. It is believed that these nicks are needed to reduce the torsional stress. These nicks are mainly produced during tran-

sition from round to elongated spermatids in the testis and occur before complete protamination of sperm nuclei. These loop domains are created and ligated by topoisomerase II during this process^(36,37).

Thus, the enzyme disturbance can result in defect in packaging of sperm DNA and may be contribute to male infertility. It is suggested that enzyme inhibitors might increase the levels of internal DNA breaks by preventing their repair and increasing their susceptibility to damage⁽³⁸⁾.

The histones should be replaced by protamines during sperm DNA packaging for more chromatin condensation⁽³⁹⁾. Human sperm has 2 different types of protamines in equal amounts: P1 and P2⁽⁴⁰⁾. Recent studies indicate that the ratio of P1 to P2 is critical for sperm's fertilization ability⁽³⁹⁻⁴¹⁾. Also, it has been shown that P2 precursors (pre-P2) play a pivotal role in maintaining this ratio. Any defect in pre-P2 mRNA translation appears to cause abnormal sperm morphogenesis, reduced sperm motility, and subsequent male infertility^(42,43). Moreover, the chromatin stability depends on the number of disulfide cross-links between thiol groups of adjacent protamine chains^(34, 44).

More recent data indicate that the stabilization of chromatin begins in the testis and continues during its passage through the epididymis⁽⁴⁴⁾. As sperm migrates along the epididymis, cysteine sulfhydryl groups in testicular sperm are progressively oxidized to disulfide bounds that increases compaction of DNA-protamine complex^(4,45,46). Disturbance at any stage of this process can lead to permanent defects in sperm chromatin.

Apoptosis

A number of studies have proposed that the presence of spermatozoa containing apoptosis signals such as damaged DNA is indicative of escaping sperm of the apoptosis process, which is referred to as "abortive apoptosis"⁽⁴⁷⁾.

Spermatogonial stem cells proliferate clonally during mitoses before undergoing the differentiation steps. This proliferation is ex-

cessive and needs a mechanism such as apoptosis to match the number of them with the supportive capacity of sertoli cells⁽⁴⁸⁾. Therefore, apoptosis is a vital mechanism to control the over production of sperm⁽⁴⁹⁾.

Pathways involving the cell-surface protein Fas may mediate apoptosis in sperm⁽⁵⁰⁻⁵²⁾. Thus, the Fas positive germ cells will be killed by apoptosis to reduce their population. The number of Fas positive spermatozoa is very small in fertile men. However, Fas-positive spermatozoa may consist half of the sperm in men with abnormal semen parameters. Thus the correct clearance of spermatozoa via apoptosis has not occurred, and the presence of spermatozoa with apoptotic markers such as Fas molecules and DNA breakage, indicates an "abortive apoptosis" in these men⁽⁵³⁾.

Another main component of apoptotic pathways are caspases enzymes⁽⁵⁴⁾. Caspases 8 and 9 are activated via Fas ligand/ Fas ligation in the inner layer of mitochondrial membrane. Following activation, they conduct a signal to their effector such as caspase 3, which activate the caspase-activated DNase resulting in degradation of sperm DNA, and apoptosis⁽⁵⁵⁾.

Finally, telomere shortening is another inducer of apoptotic pathway which can contribute to the abortive apoptosis theory⁽⁵⁶⁾.

Oxidative stress

The most common cause of sperm DNA damage is Oxidative Stress (OS)⁽⁵⁷⁻⁵⁹⁾. OS is an imbalance between the formation of Reactive Oxygen Species (ROS) and the capacity of antioxidant scavengers to neutralize or eliminate them. The ROS might damage DNA through modification or deletions of bases, frame shifts, DNA cross linkages, chromosomal rearrangement, single and double strand DNA breaks, and gene mutations⁽⁶⁰⁻⁶²⁾.

ROS are produced in sperm through leakage of electrons from the mitochondrial electron transport chain⁽⁶³⁾, NADPH oxidase⁽⁶⁴⁾ and generation of nitric oxide (NO)⁽⁶⁵⁾. The origin of ROS may be from the outside of the sperm and even from the outside of the body.

The extrinsic sources of ROS include: cigarette smoking, increased scrotal temperature, electromagnetic radiations (cell phone), and organophosphorous pesticides ⁽⁶⁶⁾.

An increased level of ROS via damaging the inner and outer mitochondrial membranes, is the main inducer of apoptosis in spermatogenic cells ⁽⁶⁶⁾.

Assay of sperm chromatin integrity

Several methods have been developed to evaluate sperm DNA or chromatin integrity ^(67,68). A number of methods only detect breakage in single or double strands of sperm DNA. In contrast other methods are based on the fact that defects in the sperm chromatin structure have been associated with increase DNA instability and sensitivity to denaturing stress. Therefore these methods provide denaturation condition and the subsequent assessment of the sperm chromatin ability to maintain its integrity. Most of these methods are based on the staining of pre-treated spermatozoa by fluorescent or non-fluorescent dye staining. Detection of stained or unstained spermatozoa may be done by light or fluorescent microscopy. Since observational evaluations by individuals have too high inter-assay and intra-assay coefficient variation ^(69,70), recently most of the evaluations particularly for fluorescent dyes, are based on automated instruments such as flowcytometry.

Acidic aniline blue staining

The Acidic Aniline Blue (AAB) stain discriminates between lysine-rich histones and arginine/ cysteine-rich protamines. This stain specifically reacts with lysine residues in nuclear histones and reveals differences in the basic nuclear protein composition of the sperm. Histone-rich nuclei of immature sperm are rich in lysine and will consequently take up the blue stain. On the other hand, protamine rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively low levels of lysine, which means they will not be stained by Aniline blue ⁽⁷¹⁾.

Results of AAB have a negative correlation with sperm chromatin integrity and male fertility potential ⁽⁷²⁾. However, there is a

controversy on the correlation between the percentage of Aniline blue-stained spermatozoa and other sperm parameters that need to be further evaluated.

Toluidine blue staining

Toluidine Blue (TB) is a basic nuclear dye used for metachromatic and orthochromatic staining of chromatin. This stain is a sensitive structural probe for DNA. Due to the cooperative nature of metachromatic stain which is indicative only in severe DNA damaged conditions, it is revealed in only poor sperm DNA integrity. Therefore, TB staining should be used in combination with other more reliable staining methods for the assessment of sperm chromatin integrity ⁽⁷³⁾.

In general, the AAB and TB are two simple and cheap methods that have the advantage of providing suitable slides for use on a light microscope ⁽⁷⁴⁾. The smears stained with the TB method can also be used for assessment of sperm morphology. However, these methods have the inherent limits of repeatability due to dye balance differences and a low number of sperm which can be reasonably counted ⁽⁷⁴⁾.

Chromomycin A3 staining

Chromomycin A3 (CMA3) is a guanine-cytosine-specific fluorochrome and indicative of poor chromatin packaged in human sperm via indirect counting of protamine-deficient sperm. Chromomycin A3 and protamines compete for the same binding sites in the DNA. Therefore, high CMA3 stained spermatozoa is a strong indicator of the defects in protamination ⁽⁷⁵⁾.

As a discriminator of ART success rate, CMA3 method has a sensitivity of 73% and specificity of 75%. Therefore, it may provide a prognosis on the success of ART ⁽⁷⁶⁾.

DNA Breakage Detection-Fluorescent In Situ Hybridization (DBD-FISH)

In this assay, sperm was placed within an agarose gel on a slide that was exposed to an alkaline condition, which converts DNA-strand breakages into single-stranded DNA (ssDNA) motifs. After neutralizing and proteins extruding, ssDNA is available for hybridization with specific DNA probes. The

probe indicates the chromatin area to be analyzed.

As DNA breakages increase, more ssDNA is created by the alkaline condition and more probe hybridizes, which leads to an increase in the fluorescence strength and surface area of the fluorescent in situ hybridization (FISH) signal. Defects in sperm chromatin packaging significantly increase the availability of DNA ligands and the sensitivity of DNA to denaturation by alkaline condition. Therefore, DBD-FISH used for in situ evaluation and quantification of DNA breakages, brings to light the structural aspects of the sperm chromatin^(77, 78).

Although this method shows structural aspects of sperm chromatin, it is expensive and time-consuming. In addition this assay has less clinical value and its results are not superior to the other methods⁽⁷⁸⁾.

In situ nick translation

The Nick Translation (NT) method measures the insertion of biotinylated deoxyuridine triphosphate (dUTP) at single strand DNA breakages that is catalyzed by DNA polymerase I. It particularly stains spermatozoa with considerable quantity of endogenous DNA breakage. The NT method shows abnormalities that have risen during remodeling of the sperm chromatin. As a result most of these anomalies have not been shown by standard semen analysis such as sperm morphology⁽⁷⁹⁾.

Application of NT assay shows an association between sperm chromatin integrity and sperm motility and morphology and to a lesser extent, sperm concentration. The NT method is used for detection of sperm DNA damage arising from causes such as heat exposures or the production of ROS following leukocytospermia and contact of sperm with leukocytes within the urogenital tract of men⁽⁷⁹⁾. The benefit of the NT method is direct labeling of the DNA breakage sites, and consequently the breakage sites are detectable at the molecular level⁽⁸⁰⁾.

Acridine Orange staining

The Acridine Orange (AO) staining as a fluorochrome measures the susceptibility of

sperm DNA to acid-induced denaturation and subsequently shifts of AO fluorescence from green (double strand) to red (single strand). AO interacts with double-stranded DNA as a monomer; however it binds to single-stranded DNA as an aggregate. The monomeric AO binding to native double strand DNA emits green fluoresces, while the denatured DNA binds to aggregated AO and produces red fluoresces⁽⁸¹⁾.

The AO assay, also named as Sperm Chromatin Structure Assay (SCSA), is a functional assay that measures sperm quality. The variation of its results between different individuals (inter-assay) and between several assays (intra-assay) for the same sample is very high. If the inter-assay coefficient variations of AO staining method were less than 5%, it is rendered as a highly reproducible technique.

To increase the accuracy and precision of AO staining results for sperm chromatin, there is a need for more expensive instrumentation such as flowcytometer to differentiate different colors and interpret the results. Also, individual subjectivity may hinder the results if fluorescent microscopy is used⁽⁸²⁾. Since the SCSA is highly constant over a long period of time as compared to the standard parameters of semen analysis, it may be applied successfully in the epidemiological studies in the field of andrology⁽⁸³⁾.

Sperm chromatin dispersion

The Sperm Chromatin Dispersion (SCD) test is based on this fact that when sperm are exposed to an acid solution prior to lysis following the removal of nuclear proteins, the DNA dispersion halos will be observed. It presents minimally in sperm chromatin without DNA fragmentation or not produced at all in sperm chromatin with fragmented DNA⁽⁸⁴⁾. The major advantage of the SCD test besides the above mentioned methods is that it does not need to detect the color or fluorescence intensity. Furthermore, the test is easy, fast, and reproducible and its results are as good as to those of the SCSA⁽⁸⁵⁾.

Comet assay

The comet assay is a single-cell gel electrophoresis for detection of DNA fragmentation in a single cell ⁽⁸⁶⁾. In this assay, sperms are stained with a Fluorochrome that binds to DNA. During electrophoresis, the movement of fragmented double-stranded DNA from a damaged sperm chromatin becomes visible as a comet with a tail ⁽⁸⁶⁾. Singh et al modified the comet assay in 1988 ⁽⁸⁷⁾ by performing electrophoresis under alkali buffers to expose alkali-labile sites on the DNA. This alteration changed the sensitivity of the assay for detection of both single and double-stranded DNA breakages ⁽⁸⁸⁾. Recently using particular software the amount of fragmentations is quantified by measuring the displacement between the nucleus "comet head" and the resulting tail. The tail lengths are used as an index for the intensity of DNA fragmentation. However, determination of both intensity and length of the tail defines it more precisely ⁽⁸⁹⁾.

This method is fruitfully used in the evaluation of DNA fragmentation after cryopreservation ⁽⁹⁰⁾. It may also prognoses the success of IVF and ICSI, and embryo quality on the base of sperm chromatin integrity particularly in couples with idiopathic infertility ^(91, 92).

The comet is a well-standardized assay that correlates significantly with TUNEL and SCSA methods ⁽⁹³⁾. It is simple to perform, has a low intra-assay coefficient of variation, and not expensive ⁽⁶⁸⁾. It is based on fluorescent microscopy, therefore, it requires a well experienced individual to examine the slides and interpret the results.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine Triphosphate-Nick End Labeling (TUNEL) assay

Terminal deoxynucleotidyl transferase mediated deoxyuridine Triphosphate-Nick End Labeling (TUNEL) assay quantifies the integration of the flurochrome or biotin labeled dUTP at single and double-strand DNA breakages in a reaction catalyzed by the Terminal deoxynucleotidyl Transferase (TdT) enzyme that is not dependent on the template.

This enzyme inserts biotinlyated dUTP or FITC-dUTP at 3'-OH end of DNA breaks to produce a signal. Intensity of signals depends on the number of DNA breaks at the head of spermatozoa. Therefore, sperm with normal chromatin integrity have only background fluorescence, while sperm with fragmented DNA (multiple chromatin 3'-OH ends) emit highly fluorescence light ⁽⁹⁴⁾.

The TUNEL assay has been usually used in andrology research related to sperm chromatin integrity and its abnormalities. It gives valuable data in numerous cases of infertile and subfertile men ^(95, 96). The flowcytometric quantification of labeled DNA 3'-OH ends in sperm head is generally more precise and reliable; but it is much more expensive ⁽⁹⁶⁾.

High-performance liquid chromatography

This method determines the concentration of 8-hydroxy-2-deoxyguanosine (8-OHdG), which is a byproduct of oxidative damage of DNA in the sperm chromatin. It is the regularly studied biomarker for oxidative damage of sperm chromatin. Along with different oxidative adducts to DNA, 8-OHdG has been selected as a representative of oxidative damage of DNA due to its high specificity, strong mutagenicity, and relative abundance in DNA ⁽⁹⁷⁾.

This method presents the most direct evidence suggesting the contribution of oxidative damage of DNA sperm in male infertility, based on the result that levels of 8-OHdG in sperm are significantly higher in infertile men than in fertile controls and have an opposite relation with sperm concentration ⁽⁹⁸⁾. 8-OHdG in sperm DNA has been shown to increase in smokers, and they inversely correlate with the intake and the seminal plasma concentration of vitamin C. If 8-OHdG modifications in DNA were not repaired, it will be mutagenic and may lead to early abortion, malformations, or malignancy in children. Furthermore, this modification could be a marker of OS in sperm, which may have negative effects on sperm function ⁽⁹⁹⁾.

Significance of sperm chromatin integrity on male fertility

It is believed that sperm chromatin integrity is correlated with male fertility⁽⁴⁷⁾, thus it has been shown that unexplained infertile men with normal routine semen parameters have a higher DNA Fragmentation Index (% DFI)⁽¹⁰⁰⁾. Evenson et al⁽¹⁰¹⁾ have shown that the DFI is the most excellent predictor of couple fertility and their ability to get conceive. There are many studies evaluating the effect of DFI on ART outcomes, especially in cases with recurrent ART failure.

The relationship between sperm chromatin integrity and IUI outcome has been shown in several studies⁽¹⁰²⁾. Host et al found no correlation between sperm DNA breakages and the fertilization rate following ICSI⁽¹⁰³⁾. In contrast several other studies have found a significant negative correlation between sperm DNA fragmentation and the ICSI results ($r=-0.23$, $p=0.017$)⁽¹⁰⁴⁾. Sun et al⁽¹⁰⁵⁾ found a negative correlation between sperm semen analysis parameters and sperm DIF.

In a new study, the proportion of sperm with fragmented DNA correlated with numbers and embryo quality, embryo development and the rate of the ongoing pregnancies. DNA fragmentation may not influence the fertilization rate following IVF or ICSI⁽¹⁰⁶⁾. However, when the patients were divided into two category according to cut-off value of 10%, the fertilization rate was significantly higher for DNA fragmentation lower than 10% (84.1 vs. 70.7%, $p<0.05$).

In a prospective study⁽¹⁰⁰⁾, Saleh et al examined the relationship between sperm DNA damage and ART outcomes in 33 couples with approved male factor infertility. They found that the sperm DFI was negatively correlated with sperm concentration ($r=-0.31$, $p=0.001$), p motility ($r=-0.47$; $p<0.001$) and normal sperm morphology ($r=-0.40$; $p<0.0001$)⁽¹⁰⁰⁾.

The current data suggest that fertilization and pregnancy rates following ICSI, are not related to the severity of the sperm defects⁽¹⁰⁶⁾. This finding has caused debate on the

usefulness of ICSI to increase fertilization, rare in patients with teratozoospermia. This may affect the quality of embryos and the resulting fetus⁽¹⁰⁷⁾. Therefore, there is a concern about the possible role of elevated sperm DNA fragmentation on embryo quality^(103,108-110), since sperm with abnormal morphology might be able to create an embryo using ICSI procedure.

However, it is suggested that low levels of DNA fragmentation can be repaired by the oocyte machinery^(111,112). In contrast high levels of DNA breaks that are further than repair, will trigger the apoptosis process followed by fragmentation of the early embryo or morbidity in later life⁽¹⁰⁵⁾. It may be the cause of early fragmentation in embryos created in IVF cycles. The rate of blastocyst stage is low in patients with high DIF, but natural selection will guarantee that most of them will abort before growing to term⁽¹¹³⁾. This may be a reason for low efficiency of fertility process and especially assisted reproductive techniques.

Recently, the importance of paternal role in early embryo development has been shown in view of that increased chromosomal damage is related to repeated spontaneous abortions⁽¹¹⁴⁾.

Repair and prevention of sperm chromatin damage

Sperm chromatin abnormality can be repaired during the period between sperm entry into the ooplasm and the beginning of the next S phase, by virtue of pre and post replication mechanisms⁽¹¹⁵⁾. Therefore, the consequences of sperm chromatin abnormality on ART outcomes, relates to the collective effects of sperm DNA breaks dosage and the ability of the oocyte to repair the preexisting damage. Though, if sperm with high level of chromatin damages are applied in ICSI or IVF cycles, the oocyte's repair competence might be insufficient or not enough. This will lead to fragmentation and a low embryo quality that is followed by *in vitro* produced embryo fragmentation and elevated rate of early pregnancy loss^(113, 116).

Sperm processing methods certainly influence selection of healthy population of spermatozoa. The chromatin integrity of processed sperm is usually more than that of un-prepared semen ⁽¹¹⁷⁾. Simple preparation techniques such as density gradient centrifugation can enrich sperm normal morphology and normal nuclear integrity ⁽¹¹⁸⁾. This improving effect of density gradient centrifugation can be the explanation for little prognostic value of sperm parameters prior to preparation in terms of fertilization and pregnancy using ARTs ⁽¹¹⁹⁾. This valuable effect corresponds with the somewhat high post-IVF fertilization rate using a simple swim-up method ⁽¹²⁰⁾.

In-vitro culture of testicular tissue has also been showed to increase the motility and recovery rate of testicular spermatozoa ⁽¹²¹⁾. The ROS production increases when spermatozoa are cultured in medium containing leukocytes, abnormal spermatozoa, and transition metals. Therefore using the processing method itself may be the cause of DNA damage. However, supporting data show that culture of testicular tissue does not increase the liability of its spermatozoa to chromatin damage. In sperm recovered from obstructive azoospermia, the proportion of spermatozoa with single stranded DNA breaks decreased considerably following 3 days of *in vitro* culture ($p=0.005$) ⁽⁴⁷⁾. Likewise, immature germ cells cultured *in vitro* for 48 hrs, make it possible to select TUNEL-negative spermatids ⁽¹²²⁾.

While ROS production is a main cause of sperm chromatin damage, antioxidant therapy may be the major approach to protect sperm chromatin integrity. When Ascorbic Acid (600 mmol/l), Alpha-tocopherol (30 and 60 mmol/l) and Urate (400 mmol/l) are added to culture media, it provides considerable protection ($p<0.001$) from DNA damage following exposure to X-irradiation. Thus, supplementation of culture media with antioxidants individually, can beneficially influence the sperm chromatin integrity ⁽¹²³⁾.

Plant derived compounds of Genistein and Equol (Isoflavones) have antioxidant activity, and so a function is suggested for them in the

treatment of male infertility. In comparison with Ascorbic acid and Alpha-tocopherol, Genistein is the most potent antioxidant, followed by Equol, Ascorbic acid and Alpha-tocopherol in culture medium. Genistein and Equol in combination are more protective to neutralize the oxidative stress. According to above results, these compounds also may have a role in antioxidant protection sperm chromatin integrity and preventing from DNA damage ⁽¹²⁴⁾.

In addition, several studies showed that administration of antioxidant supplement such as Ascorbic acid, Alpha-tocopherol, Beta carotene, Retinol, Coenzyme Q (Q₁₀), etc. in combination with drugs such as folic acid, zinc sulfate, carnitine, etc can improve the routine parameters of semen, sperm chromatin integrity and the outcome of ARTs ⁽¹²⁵⁻¹³⁰⁾.

Conclusion

It has recently been accepted that sperm chromatin integrity is an independent index of sperm quality and has better diagnostic and prognostic capacities in association with routine semen analysis results for both *in vivo* and *in vitro* fertility. Although there are numerous methods to evaluate sperm chromatin integrity, each procedure needs to be studied more and standardized for routine use in diagnostic andrology lab. However, all of these methods destroy sperm during the evaluation process. Introducing methods with some effect on whole sperm integrity will improve outcomes of ARTs using spermatozoa with approved chromatin integrity. The results may help the physicians to counsel infertile couples referred for ART in a better manner. In addition, sperm preparation techniques and *in vitro* culture of spermatozoa in special conditions, may develop its quality in men having high percentage of DFI.

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