

**Review article**

# Sperm chromatin assay and its' implication in male sub-fertility: review article

Rabab Zahir Al-Yasiry <sup>1</sup>, Sahbaa Hafedh Sagban <sup>2</sup>, Muhjah Falah Hassan <sup>3\*</sup>, Pallav Sengupta <sup>4</sup> and Sulagna Dutta <sup>5</sup>

<sup>1</sup>Department of Anatomy, Histology and Embryology, College of Medicine, Babylon University, Hilla, Iraq.

<sup>2</sup>Department of Anatomy, Histology and Embryology, College of Medicine, University of Kerbala, Karbala, Iraq.

<sup>3</sup>Department of Anatomy, Histology and Embryology, College of Medicine, University of Kerbala, Karbala, Iraq.

<sup>4</sup>Department of Biomedical Sciences, College of Medicine, Gulf Medical University, Ajman, UAE.

<sup>5</sup>Basic Medical Sciences Department, College of Medicine, Ajman University, Ajman, UAE.

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**\*Corresponding:**

[muhjah.f@uokerbala.edu.iq](mailto:muhjah.f@uokerbala.edu.iq)

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**Abstract**

Defect in sperm parameters decides the fertility potential of sub-fertile men and adversely correlates with pregnancy rate both in vivo and in vitro. Classical seminal fluid analysis is of a limited value in describing mans' fertility status. This review is aimed to investigate the role of sperm chromatin assay and DNA damage in male sub-fertility. A clinical evidence has showed that sperms DNA damage has a negative impact on reproductive outcomes and the sperms of sub-fertile men substantially exhibit more DNA damage than those of fertile one in such a level that embryo development and pregnancy rate are impaired.



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## 1. Introduction

### Structure of Sperm Cell

The sperm is a specialized cell with a unique structure and function to carry the 23 male chromosomes and the centrosome (cell center) to the oocyte during the process of oocyte fertilization [1]. The centrosome of the sperm cell is considered as a machine for cell division during mitotic process. The sperm cell consists of a head, neck, mid-piece and tail [2] Figure 1.

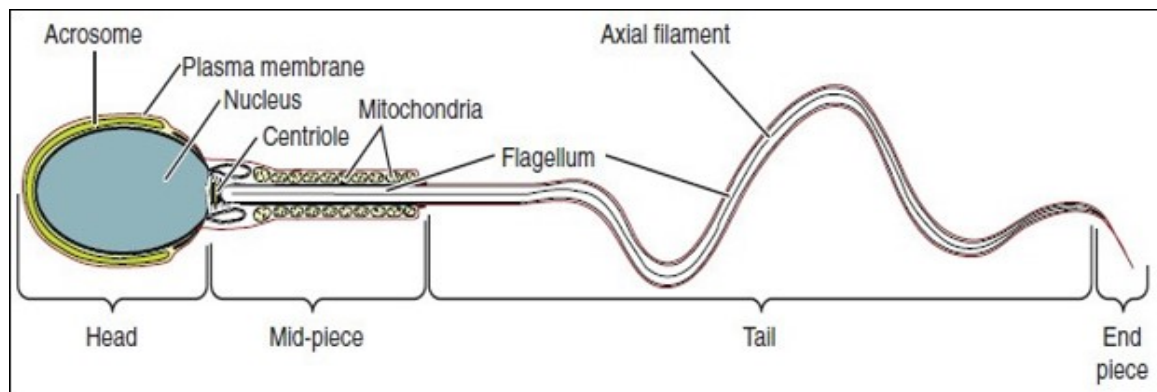


Figure 1: Sperm cell structure

Regarding its' structures; head (outer plasma membrane (PM), acrosome) and nucleus which consists of a highly condensed DNA. The neck contains the centrosome with a single functional centriole. The mid-piece and the tail both are responsible for sperm motility, the tail mainly consists of an axoneme, while the mid-piece contains mitochondria which is the energy source for sperm motility [3].

### Physiology of the mature sperm

Within aqueous environment, the velocity of the sperm is about 1 to 4 mm/min. Sperm activity is usually enhanced in a neutral and slightly alkaline medium. The highly acidic media will destroy it. Sperm activity and metabolism are increased by temperature due to shortening the life span of sperms. However, sperms have the ability to survive for few weeks in the suppressed conditions within the genital ductal system of the testes [4].

During sexual intercourse, millions of sperms are ejaculated in the vagina of the female and from them only a single sperm will fertilize the oocyte. The selection of the fertilizing-sperm occurs at multiple sequential steps, when the ejaculated sperm have to negotiate environments that due to their physical, chemical, and biological properties act as barriers. The lack of ability of sperm cell to undergo active transcription and translation, sperms usually survive within female genital tract without any reparative mechanisms [5].

During ejaculation, sperms are usually more liable to oxidative damage due to physical stress together with contractions of the female tract, this might make them sometimes targeted by of the female immune system. The acidic intra-vaginal environment is not favorable for long time sperm viability and survival, so only those that have the ability to pass rapidly within less hostile cervical canal remain viable [3].

Cervical mucus reduces sperm progression and this process is accentuated by the complex architecture of the cervical canal. As sexual intercourse and ovulation are not always biologically compatible, so to increase the chance of pregnancy, the potentially fertilizing-sperm must be retained in the cervical crypts and gradually released up toward the uterus. It has been suggested that fertilization may take place when intercourse occurs up to 5 days before ovulation which needs a functionally competent sperms to be sufficient for the long term survival [6].

Within one minute of sexual intercourse, the semen coagulates forming a loose gel which then enzymatically degraded within thirty minutes or sometimes up to one hour. Semen coagulation helps to entrap the sperms at the cervical opening thus protecting them from the hostile environment of the female vagina [3]. In addition to buffering mechanism, seminal plasma contains protective constituents which coat the sperm and prevent their tackling by female immune system. This protective mechanism is most efficient when sperms are still within seminal plasma and gradually decreased when sperms are separated from seminal plasma. Cervical mucus constitutes a bigger obstacle to poorly-motile sperm than vigorous, progressively motile, morphologically normal sperm and thus might considered as a tool of sperm selection [7]. To overcome the cellular immune responses, a large number of sperms to female genital tract should be inseminated. Before the fertilizing-sperm reaches the oocyte, it must penetrate a couple of layers; cumulus oophorus and zona pellucida (ZP). Receptor-mediated reactions with molecules found in these layers allows sperm passage through the oocyte. Progesterone in the cumulus oophorus and the ZP3 ligand-protein in the zona pellucida considered as a major contributors in sperm penetration to the oocyte [4].

The last phase of fertilization is called sperm capacitation. The capacitation is a necessary prerequisite for the acrosome reaction which is essential prerequisite for zona pellucida penetration. These steps subjected the sperms to a further selective process, since sperm which lacks functional receptors and signal transduction pathways fails to reach to the surface of oocyte [8].

### Sperm capacitation

Capacitation of the sperm makes the sperm able to penetrate the oocyte. Despite of acquiring their maturity upon leaving the epididymis, their major activity is held by many inhibitory factors which are secreted by the epithelium of male genital duct. So, they are unable to do their job in fertilizing the oocyte immediately following ejaculation to the seminal fluid. Activation of the sperm (being able to fertilize the oocyte) is occurred within female genital tract. These activation processes are collectively named capacitation of the sperm. It normally needs 1 to 10 hours. Capacitation involves changes in sperm plasma membrane by shedding of proteins and cholesterol and this might reduce affinity for endosalpigeal epithelium [9] and Figure 2.

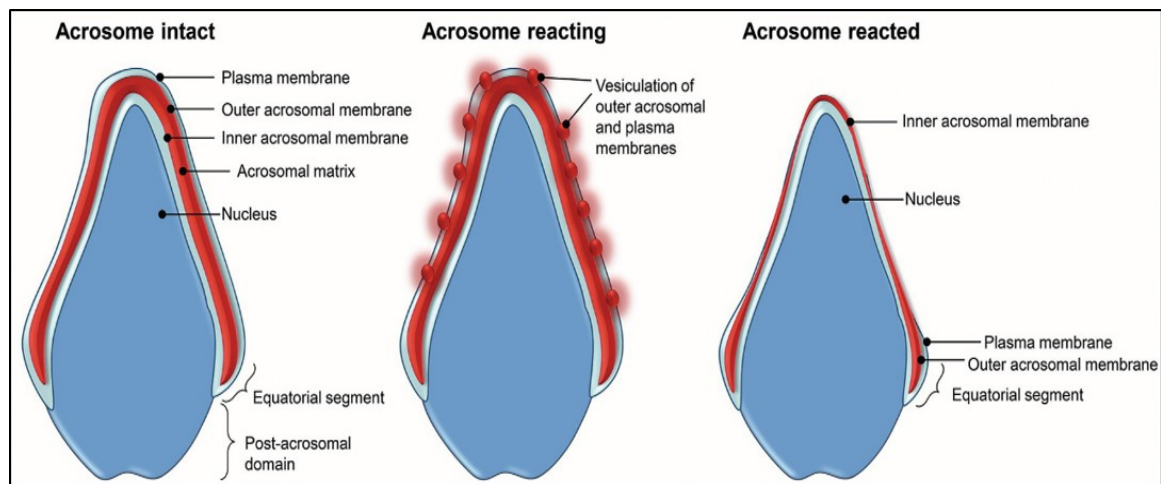


Figure 2: Sperm capacitation

#### These changes are summarized in the following steps:

1. The inhibitory factors that suppress sperm activity in the male genital ducts are washed away by female tubal and uterine fluids [10].
2. Within the male genital tract, sperms are exposed to seminiferous tubules floating vesicles. A large amount of cholesterol is continually added to the plasma membrane which overlies the acrosome thus providing a strength for the acrosome and preventing the release of its enzymes. Following ejaculation and when the sperms are deposited in the vagina, the sperms leave the cholesterol vesicles and swim away toward the uterus. So the cholesterol is gradually lost in the next few hours. This resulted in a gradual weakening of the membrane at the head of the sperm [7].
3. Permeability of the plasma membrane to  $Ca^{++}$  is also documented, an abundant amount of  $Ca^{++}$  entered enhancing the activity of sperm tail, resulted in a strong whiplash movement from a previous weak undulating movement [11]. Alteration in  $Ca^{++}$  across the acrosome plasma membrane also leads to release of acrosomal enzyme [4].

#### Release of acrosomal enzymes; Acrosome reaction

An abundant amount of hyaluronidase and proteolytic enzymes are stored in the acrosomal region of sperm head. Hyaluronidase depolymerizes the hyaluronic acid polymers which are responsible of keeping the granulosa cells surrounding the oocytes attached to each other. While the proteolytic enzymes hydrolyze proteins in the tissue that adhere to the oocyte [10].

Within few minutes, the released-enzymes provide a penetrating pathway allowing the passage of the sperm head through the zona pellucida toward the center of the oocyte. Within another half hour, a fusion between the plasma membranes of the sperm head and that of the oocyte is started forming a single cell. The remaining sperms are either phagocytosed by the epithelium of the female genital tract or expelled into the peritoneal cavity where the phagocytic process takes place [5].

#### Sperm chromatin

A traditional method for assessing male fertility potential is by measuring the count, motility and morphology of the sperms in the ejaculated semen (seminal fluid analysis). It has been suggested that the integrity of sperm DNA may be used as a predictor of male fertility and provides an additional support to the traditional semen analysis [12]. The main function of the sperm is delivery of paternal genome to the maternal cell (oocyte). The DNA is found in the head of a mature sperm in a condensed, compacted insoluble structure [13]. The tightly condensed structure of the sperm chromatin is due to unique correlation between the DNA and nuclear proteins which entirely resulted in different quality of DNA packaging in sperm nuclei [13]. The mature sperm is produced by a specialized event; sequential alteration of cytoplasmic architecture throughout a series of meiotic and mitotic divisions, histones exchange with transition proteins and replaced by protamines forming a tightly compacted chromatin at the later stages of spermatogenesis and throughout the spermiogenesis [14]. This packaging makes the chromatin of the sperm approximately 6 times more compacted than that in somatic chromosomes. More than 85% of the sperm DNA is found bounded by protamine forming DNA-protamine complexes having a more compaction than DNA-histamine complexes [15]. A further reduction in the chromatin volume occurs in the epididymis by a cross-linking between protamines [16].

#### Multiple benefits has been provided from DNA packaging:

1. Minimization of the size of the sperms during their journey in male and female reproductive system.
2. Minimization of sperm destruction by exogenous substances before fertilization.
3. Keeping the genome of the sperm inactive transcriptionally.

Thus, the sperm DNA has been kept protected during sperm passage within the male and female genital tracts [17]. De-condensation of sperm chromatin is started post fertilization. Any abnormality in the sperm DNA such as protamine deficiency and retention of more than 15% histones forming a less-condensed chromatin. Moreover, the sperm contains another kind of protamine, called P2, that is deficient in cysteine [18]. The sperm which displayed an altered P1/P2 ratio are normally more susceptible to stressful conditions and usually correlated with sperm DNA fragmentation [19].

## Origin of sperm chromatin abnormalities

In the final stages of spermatogenesis and as a result of inhibition of DNA repair systems and loss of the cellular apoptotic machinery system, the sperm cells are highly susceptible to DNA fragmentation and the progressed stages of germ cell differentiation cannot be deleted [14]. So, the consequence is that the ejaculated sperms may show a genetic damage which could be affects the zygotes which are formed by the fertilizing-sperm. This damage should be corrected, preferably before the 1st mitosis. It has been showed by several studies that oocytes and earlier stages embryos have the ability to repair sperm DNA damage and errors which may occur during post-fertilization DNA corrective period can lead to mutations that may affect fetal development and sometimes extend to childhood period [20].

## Main patho-physiological processes that underlie sperm DNA damage

### Defective packaging of sperm chromatin

Abnormal packaging tends to be associated with nuclear DNA damage [21] and temporary relief of torsional stress mandates DNA breaks. Thus, the process of chromatin repackaging needs endogenous nuclease activity that is able to create and ligate breaks which occurs in a sensitive and critical steps. Thus, non-ligated breaks are not accepted and maturation-associated DNA breaks must be repaired totally. Failure of repairing and correction of the transient breaks (either due to increased topoisomerase II activity or a lack of its' inhibitors), fragmentation of DNA will result. Sub-optimal condensation during epididymal making the resulted sperms more candidate for DNA fragmentation [21].

### Abortive apoptosis

Failure of sperm cells to respond to any of the signals that control the programmed cell death may occur due to inability of sperms to create proteins. Apoptosis; programmed cell death; is initiated within the seminiferous tubules by highly specialized Sertoli cells and influence 50-60% of sperm germ cells that progress to 1<sup>st</sup> meiotic division [22].

Initial apoptotic pathway is started in spermatogonia and spermatocytes and mediated usually by Fas protein [23]. Fas is a type I membrane protein and a member of tumor necrosis factor (TNF)/nerve growth factor receptor (NGFR) family. By an interaction between Fas and Fas ligand in sertoli cells, cell death via apoptosis is initiated and resulted in reduction of the size of the germ cell population to such a number that Sertoli cells able to deal with and support.

Some men with defective semen parameters usually have a significant number of sperms that bear Fas, suggested that these cells are the final result of an incomplete apoptotic cascade [23].

### Oxidative stress

As a result of high proportion of poly-unsaturated-fatty acids and inadequate antioxidant protection, sperms are so liable to oxidative destruction forming reactive oxygen species (ROS). ROS have a significant physiological role; alteration of gene and proteins that are essential for sperm replication, specialization, and normal function. Although, little amounts of ROS are mandatory for certain sperm functions (capacitation and acrosome reaction), increased level is negatively affect sperm progressive motility and morphology. They may be generated due to uro-genital tract infections, neutrophilic and macrophages infiltration, radiation, chemotherapy and smoking [24]. Their production is under the control of seminal antioxidants [14]. So, the harmful effects exhibited when their production is so excessive to be overcome by the seminal plasma antioxidant capabilities. Oxidative stress (OS) causes peroxidative damage in PM and DNA fragmentation [25].

### Deficient recombination

Crossing-over which takes place during meiosis includes a programmed introduction of DNA double-strand breaks (DSBs) by specific nucleases [26] which have to be repaired at the end of 1st meiosis. Normally, a recombination checkpoint in meiotic prophase prevents 1st meiotic division to continue till full correction of DNA or ablation of defective spermatocytes. However, a persistent sperm DNA fragmentation in ejaculated semen may occur as a result of a defective checkpoint.

## Other contributing factors to sperm DNA damage

Advanced-age males [27] and young males with malignancy may have abnormally bad semen parameters and DNA fragmentation even prior to initiate cancer therapy. A recent data from patients with testicular cancer showed that radiotherapy induced temporary sperm DNA damage which evenly might persist up to 3–5 years later [28].

Smoking has a negative impacts on semen parameters causes a reduction in sperm count, motility and an enhancement of morphologically abnormal sperms with DNA damage by increasing the production of leukocyte-derived ROS [29].

Also, leukocyte-spermia related to oxidative stress and subsequent sperm DNA damage may occur due to genital tract infections and inflammation ,organophosphates poisoning (pesticide) and exposure organo-chlorine pollutants [20].

Varicoceles also a considerable factor of oxidative stress and sperm DNA damage with a documented improvement in sperm DNA integrity following varicocele repair [30].

A significant deficiency in follicle stimulating hormone (FSH) may cause sperm chromatin defects. A febrile illness and direct exposure of the testis to heat have been found to cause an elevation in the histone/protamine ratio and DNA defect) [31].

Eventually, sperm preparation techniques (density gradient centrifugation and swim-up methods) during assisted reproduction techniques (IUI, IVF and ICSI) involves a frequent exposure of the sperm to a high-speed centrifugation to separate the sperms from the seminal plasma, (which is considered as a protective antioxidant media) may lead to increased sperm DNA damage due to production of high level of ROS [32].

## Indications for sperm chromatin assessment

Over the past decades, the association among sperm DNA integrity damage, mens' fertility potential and effects on both natural and assisted conceptions have been fully investigated.

At time of intra cytoplasmic sperm injection (ICSI), only viable, morphologically normal sperms are isolated for injection. It was showed that the selected morphologically normal sperm may have a hidden DNA damage. And depending upon morphology is sometimes considered as a wrong method prior to oocyte injection [13].

## Diagnosis of male infertility

Sperm DNA damage decreases the chance of natural conception in the affected couples. There is a significant variation in cutoff levels of sperm chromatin defect between fertile and infertile male [33]. Moreover, sperms of infertile men are highly sensitive to damaging substances like oxidative species and radiation causing varying degree of sperm DNA damage. There is nearly no chance of getting pregnancy when the % of sperms with chromatin abnormality more than 30% as detected by sperm chromatin structure assay (SCSA), or 20% as detected by TUNEL [34]. This indicates that sperm chromatin assay may disgnose a concealed sperm defect in infertile males who labeled under the term "unexplained infertility" depending on their normal traditional seminal fluid analysis [33].

## Assisted reproductive technologies

It has been suggested that by using a sperm with DNA damage during ART procedures, the chance of genetic defect in the developing embryo may be increased [35]. These genetic defects are responsible for poor embryogenesis and impaired implantation [36]. Most of the studies exhibited a significant negative correlation between sperm DNA damage and embryo quality, fertilization rates and blastocyst formation after ICSI [37].

## Embryonal loss

By investigation of the couples with recurrent abortion, it was showed that the percentage of sperm with DNA damage is significantly higher in the males of those couples when compared with the general population or fertile donors [38]. It also associated with a high incidence of repeated pregnancy loss and disease in offspring, like childhood cancers and autism [25]. In a research done by [38] it was concluded that couples with a DNA fragmentation index (DFI) of 26% were able to get pregnant, but could not keep the pregnancy which ends with recurrent pregnancy loss. Proved a correlation between high DFI and embryo chromosomal abnormalities [39, 40].

## Cancer patients

Some evidences from laboratory researches on animals indicate that testicular radiation exposure may lead to sperm DNA fragmentation [39].

## Evaluation of sperm nuclear DNA damage

Different procedures can be used to assess the quality of the sperm chromatin and to examine any defect or immaturity. Many procedures include simple staining or more complicated techniques are shown in table 1 and Figure 3.

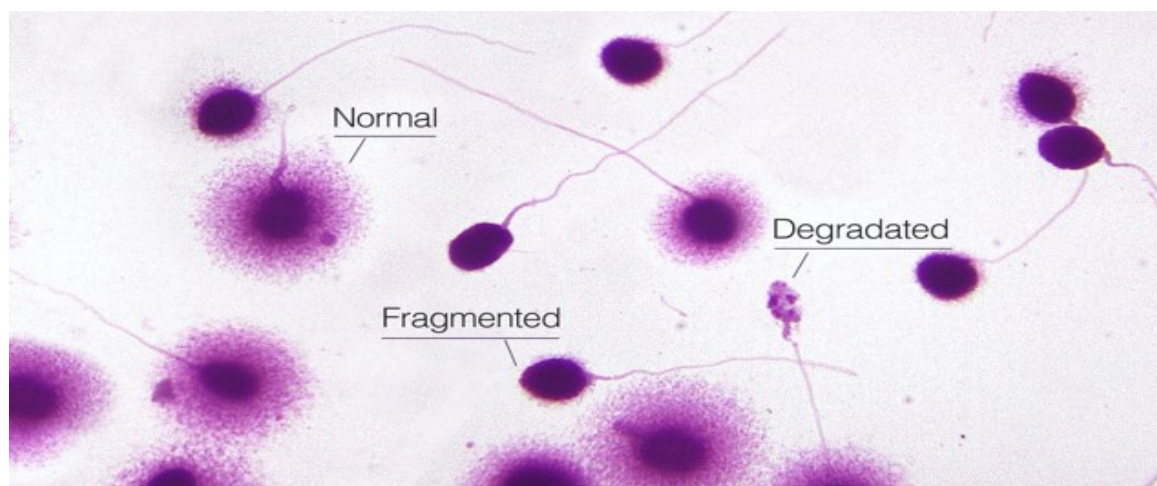


Figure 3: DNA fragmentation test (Halo test)

**Table 1:** Various methods for assessing sperm chromatin [20]

Assay	Parameter	Method of analysis
Acidic aniline blue	Nuclear maturity (DNA protein composition)	Optical microscopy
Toulidine blue stain	Nuclear maturity (DNA protein composition)	Optical microscopy
Chromomycin A3	Nuclear maturity (DNA protein composition)	Fluorescent microscopy
DNA breakage detection (FISH)	DNA fragmentation (ssDNA)	Fluorescent microscopy
In situ nick translation	DNA fragmentation (ssDNA)	Fluorescent microscopy Flow cytometry
Acridine orange	DNA denaturation (acid)	Fluorescent microscopy Flow cytometry
Sperm chromatin dispersion	DNA fragmentation	Fluorescent microscopy
Comet (neutral)	DNA fragmentation (dsDNA)	Fluorescent microscopy
Comet (alkaline)	DNA fragmentation (ssDNA/dsDNA)	Fluorescent microscopy
TUNEL	DNA fragmentation	Fluorescent microscopy Flow cytometry
Sperm chromatin structure assay	DNA denaturation (acid/heat)	Flow cytometry
8-OHdG measurement	8-OHdG	High-performance liquid Chromato – Graphy

## Strategies to reduce sperm DNA damage

### Choosing appropriate sperm preparation methods

As mentioned previously, most of the widely used methods; density gradient centrifugation, swim-up, and glass wool filtration, yield sperms with higher DNA integrity [41]. Thus, sperm preparation should be aimed to minimize damage to the sperms as little as possible. This could be done by applying some simple precautions such as:

1. The dilution of the samples should be done gradually particularly with cryopreserved sperms.
2. The tests should performed at 37°C and minimization of temperature changes.
3. Centrifugation should be used only when necessary and carried out at the lowest possible speeds.
4. Controlled exposure to potentially toxic and harmful materials like Plastic glassware and media which should be checked for toxicity to sperm and contact with gloves, as it may immobilize the sperm [32].

### Antioxidant treatments

Researches tried to study the possibility of antioxidant treatments to manage male sub-fertility with a significant improvement in clinical conception and implantation rates in those with high sperm DNA damage when treated with antioxidants before ICSI have been documented [42].

### Magnetic cell separation

A primary criteria of apoptosis is the externalization of the phospholipid phosphatidylserine (PS) which is a component of the inner PM. PS has been discovered on the surface of apoptotic spermatozoa [43]. It has been discovered that PS show a high affinity for the phospholipid-binding protein annexin V. This finding led to the development of magnetic activated cell sorting (MACS) with annexin V microbeads to isolate dead and apoptotic-like spermatozoa before selecting single spermatozoa for ICSI [44] Figure 4.

### High-magnification ICSI

Non-invasive techniques have been newly applied to enhance the ICSI success rate [45].

Intra cytoplasmic morphologically selected sperm injection (IMSI) depends on the selection of normal nuclear spermatozoa vacuoles free, using computer-enhanced digital microscopy at Very high magnification 6000 times than magnification used during ICSI [46].

### PICSI (physiologic ICSI)

The physiologic ICSI (PICSI) technique is applied to choose mature sperm with normal nuclei. In vivo fertilization hyaluronic acid (HA) has a major function in selecting only the mature sperm [47]. During PICSI, mature spermatozoa will bind permanently to HA in vitro. These spermatozoa have finished PM remodeling, cytoplasmic extrusion, and nuclear maturity. Therefore, the mature spermatozoa may have a

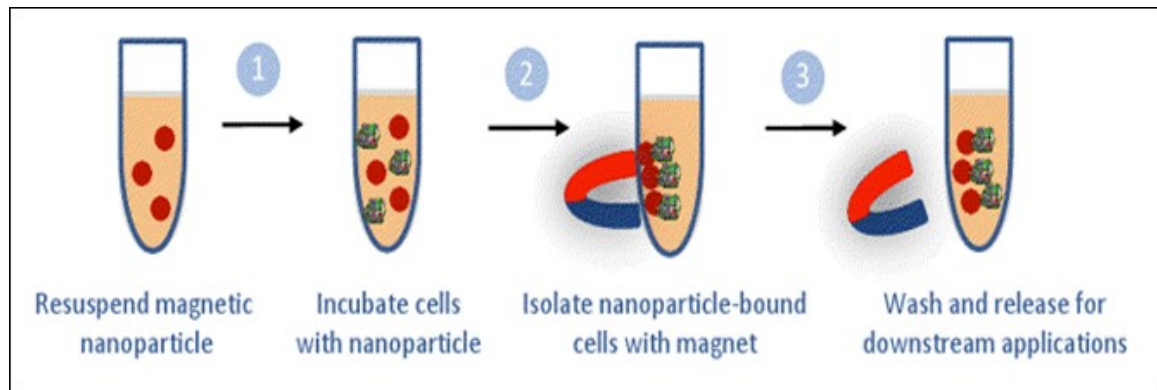


Figure 4: Magnetic sperm cell separation (MAC)

normal protamine and healthy DNA. HA sperm selection for ICSI may lead to higher fertilization rates, with increased implantation and pregnancy rates [47] from Figure 5.

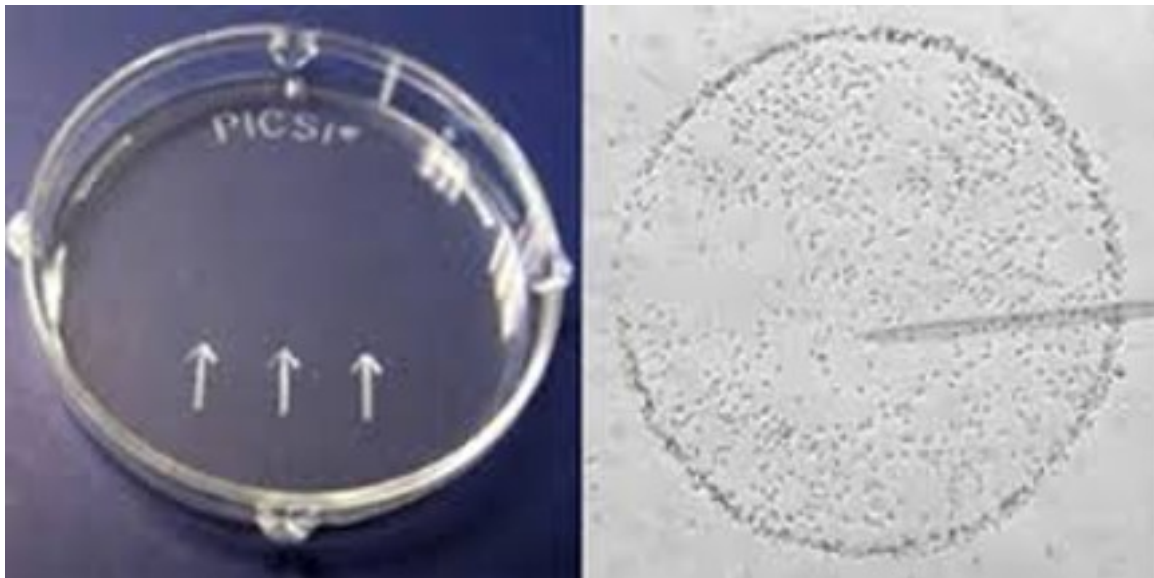


Figure 5: PICSI Dish

## 2. Conclusion

A clinical evidence has showed that sperms DNA damage has a negative impact on reproductive outcomes and the sperms of sub-fertile men substantially exhibit more DNA damage than those of fertile one in such a level that embryo development and pregnancy rate are impaired.

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