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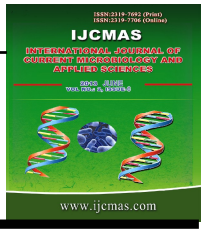


**Original Research Article**

# Assessment of sperm DNA integrity by Toluidine blue staining technique in infertile patients and its relation to cryopreservation

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**Keywords**

Infertility; Male Factor; Toluidine blue; DNA damage; IVF programs.

**A B S T R A C T**

Infertility id defined as inability to conceive after a year of regular unprotected intercourse. More than 30-40% of infertility cases are attributed to the male factor alone. Thirty semen samples were obtained at the fertility center of Billroth hospital for patients undergoing routine semen analysis for infertility. Then all the 30 samples were analyzed with toluidine blue (TB) to assess sperm DNA integrity. Out of 30 patients 6 have < than 20million/ml, 10 has < than 50% motility and the % of DNA damage is < 30% for 10 patients. If the count and motility was effected, there is a significant reduction of normal sperm parameters and the % of DNA damage is higher. Cryopreservation means storage at low temperature (196°C in liquid nitrogen), Cryopreservation of human spermatozoa is used extensively in artificial insemination and IVF programs. Freezing semen, has no effect on the of DNA denaturation. Cryopreservation is one important method to store the sperm for future use.

## Introduction

Fertilization involves direct interaction of the sperm and ooeytes fusion of cell membranes and union of male and female gamete genome. The completion of this process and subsequent embryo development depend in the part on the inherent integrity of the sperm DNA (Ahmadi *et al*., 1999). Fertility issue affects the ability to become pregnant. Infertility is a major problem today. As recently as 1980 only an estimated 10-15 couples were affected. Now most survive puts the percentage of couples remaining child less involuntarily at above 20% in developing countries. Infertility is defined regular unprotected intercourse. Most coupes are advised to seek help after trying for a year but some with more complex or pre-existing problems are advised to-do so earlier. Fortunately medical advance have come up with answer to most infertility problem.

### Fertility Test

Some of the more common investigation of fertility tests is Blood Test, Vaginal Swabs, Semen Analysis, Semen Culture, Ultra Sound Scans and Laparoscope. The average chance to conceive for a normally

fertility couple having regular, unprotected intercourse is approximately 25% during each menstrual cycle. In most couples conception occur within about 10% of couples of childbearing age. Infertility is not just a women's concern. A problem with the sole cause or contributing causes of infertility in about 40% of infertility couples. About one-fourth of infertility couples have more than one cause or factor related to their inability to conceive. About 10-15% of couples have no identification cause for their infertility after medical investigation.

## Female Infertility

The defect in female or inability to conceive by a female is called female infertility.

## Male Infertility

The inability of male to produce an offering is called male infertility. More than 30-40% of infertility cases are attributed to the male factor alone i.e., fertility problem in men. A list of risk factor related to male infertility: History of prostatitis of genital infection, Testicular trauma or torsion, History of precocious puberty, Exposure to toxic substances or hazards on the job, such as lead, mercury, x-ray, etc., Cigarette or marijuana smoke, Heavy alcohol consumption, Exposure of the genitals to high temperature, Hernia repair, undescended testicle, Prescription drugs for ulcers or psoriasis, DES taken by mother during pregnancy, Mumps after puberty.

## Semen analysis

According to WHO the following criteria for normality for a semen sample are commonly used.

WHO Criteria of semen report

| **Volume** | **2.0ml or more** |
|---|---|
| pH | 7.2-7.8 |
| Sperm concentration | 20 million/ml |
| Total sperm count | 40 million |
| Motility | 30% or more |
| Morphology | 50% or more |
| Viability | 50% or more |
| WBC | < 1million |
| Zinc | 2.4 more per ejaculate |
| Citric acid | 52 more per ejaculate |
| Fructose | 134 more per ejaculate |
| MAR test particles | Fewer than 10% spermatozoa with adherent |
| Immunobead test | Fewer than 10% spermatozoa with adherent |

Although fertile men as a group have higher mean sperm parameters (concentration, motility and morphology) than do infertile men, there is significant overlap between these groups (Evenson *et al.*, 1999). Conventional semen parameters fall short. There is now some evidence to suggest that markers of sperm DNA integrity may be better measures of male fertility potential than conventional measures, the study of sperm DNA damage is particularly relevant in an era where advanced forms of assisted reproductive technologies are frequently used.

Indeed, there appears to be a threshold of sperm DNA damage (i.e., DNA fragmentation, abnormal chromatin packaging, and protamine deficiency) beyond which embryo development and pregnancy are impaired (Cho *et al.,* 2003). There is now clinical evidence to show

that damage to human sperm DNA may adversely affect reproductive outcomes and the spermatozoa of infertile men possess substantially more DNA damage than do spermatozoa of fertile men (Spano *et al.,* 2000; Zini *et al.,* 2001; Kodama *et al.,* 1997). Unlike the relatively loose structure of chromatin (DNA and nuclear proteins) in somatic cells, sperm chromatin is tightly compacted because of the unique associations between the DNA and sperm nuclear proteins (predominantly) highly basic proteins known as protamines (Ward *et al.,* 1991). during the later stages of spermatogenesis, the spermatid nucleus is remodelled and condensed, which is associated with the displacement of histones by transitions proteins and they by protamines (Steger *et al.,* 2000). The DNA strands are tightly wrapped around the protamine molecules (about 50 kb of DNA per protamine), forming tight and highly organized loops (Brewer *et al.,* 1999).

Although the bulk of the sperm DNA is in the nucleus, a small fraction is of mitochondrial origin. The sperm mitochondrial DNA is a small, circular DNA that is not bound to proteins (Anderson *et al*., 1981). Mitochondrial DNA exhibits a high rate of mutation. Sperm motility is related to the mitochondrial volure within the sperm midpiece, and mutations or deletions in the mitochondrial DNA have been associated with reduced sperm motility (Kao *et al.,* 1998). Sperm DNA damage is clearly associated with male infertility (and abnormal spermatogenesis0, but a small percentage of spermatozoa from fertile men also possesses detectable levels of DNA damage an important subset of infertile men (about 5%-15%), but not of fertile men, possess a complete protamine deficiency (Carrel *et al.,* 2001).

In-vivo fecundity decreases progressively when >30% of the spermatozoa are identified as having DNA damage. Several methods are used to assess sperm chromatin DNA, which is considered an independent measure of sperm quality that may yield better diagnostic and prognostic approaches than standard sperm parameters (concentration, motility and morphology). Sperm nuclear chromatin abnormalities DNA damage could occur at the time of or result from DNA packing at spermogenesis. One are of research that has been studied intensely is the integrity of the nucleus of mature ejaculated spermatozoa. It has been shown that, in men with abnormal sperm parameter, the DNA is more likely to possess strand break. Alternatively, it could be the result of free radical-induced damage or a consequence of apoptosis.

Sperm DNA damage can be measured directly (fragmentation, oxidation) or indirectly (sperm chromatin compaction). Direct assessment of DNA damage can be obtained by means of single-cell gel electrophoresis assay or "Comet" assay (electrophoresis causes DNA fragments to migrate away from the central DNA core, revealing a "comet"), terminal deoxynucleotidy tranferase-mediated dUTP-nick end-labeling or "TUNEL" assay (the ends of fragmented DNA are tagged) and liquid chromatography to measure DNA oxidation levels (Aranvindan *et al.,* 1997). DNA damage can also be assessed indirectly by means of sperm chromatin integrity assays and by evaluation of nuclear protein levels (Erenpreiss *et al.,* 2001). Sperm chromatin integrity assays include slide-based sperm nuclear protein stains (e.g. aniline or toludine blue (detects histones), CMA3 (detects under protamination)

(Bianchi *et al.,* 1993). TB is a classic nuclear (cationic) dye used for external metachrmatic and orthochromatic staining of chromatin, which overall is negatively charged (Sylven *et al.,* 1954; Erenpreisa *et al.,* 1992). Thus, orthochromatic (blue) staining is the result of monomeric dye forms characteristic for either low dye concentrations or low accessibility of sites on the chromatin. Metachromatic (purple) staining, on the other hand, is the result of polymetric dye forms characteristics of cooperative binding to the DNA phosphate residues. The first situation corresponds to highly packaged chromatin of mature sperm cells with their very low stainability by external dyes (Darzynkiewics *et al.,* 1990) The second situation arises when chromatin proteins are more loosely electro statically bound to the DNA and can dissociated from it easily, and DNA stains (e.g., acridine orange detects denatured or single-stranded DNA)

The integrity of the sperm DNA may be tested to predict pregnancy outcomes in couples who do not know their fertility potential (i.e. first pregnancy). Couples in whom the man has a high percentage of spermatozoa with DNA damage have very low potential for natural fertility and will have to wait a long time before conceiving (Loft *et al*., 2003). Couples whose pregnancy resulted in miscarriage demonstrate a trend toward poorer sperm DNA integrity compared with highly fertile couples.

### Cryopreservation of sperm

Cryopreservation (Gr. kryo means frost), It means storage at very low temperature such as over solid carbon dioxide (-79°C), in deep freezers (-80°C), in vapor phase nitrogen (-150°C) or in liquid nitrogen (-196°C). During the last 50 years the field of assisted reproductive technologies (ARTs) has under gone a tremendous advancement. In the context of the cryobiology of reproductive cells (i.e., overain tissue) establishment of methods and application for spermatozoa has the longest and is the most widely used in human reproductive medicine (HRM) and in agriculture. Polge *et al.,* (1949) used glycerol as a cryoprotective agent (CPAs). Although other CPAs (dimethyl sulfoxide, ethylene glycol, methanol, propylene glycol) have emerged and in some cases are more effective, glycerol remain one of the most commonly used CPAs. This finding gave rise to the general uses of CAPs for many different reproductive cell and tissues.

Cryopreservation of semen is routinely used in a variety of circumstances including assisted reproductive, pre-radiation, chemotherapy, treatment as fertility insurance for men under going vasectomy and for stage of donor semen until seronegativity for HIV and hepatitis in conformed. It is used for storage of sperm retired from azoospermia patients who have undergone testicular sperm extraction or pereutaneous epidermal sperm aspiration of testicular and or epididymal sperm ensure sperm are available for multiple ICSI treatment and other ARTs techniques from a single biopsy (Tournaye, 1999)

## Materials and Methods

### Material

The human semen was collected from different age groups for infertility studies.

### Methods of semen collections

Thirty semen samples were obtained at the

fertility centre of Billroath Hospital for patine under going routine semen analysis for infertility. Samples were obtained by masturbation after the recommendation time of sexual abstinence. Standard semen parameters (volume, concentration, count, motility, morphology etc) were measured according to World Health Organization guidelines (WHO, 1999). All the 30 samples were analyzed with toluidine blue (TB) to assess sperm DNA integrity.

**Semen analysis**

The semen samples from 30 patients of infertile males subjected to analysis of microscopic, physical and morphological characters of sperm.

**Microscopic examination of semen**

After the collection of sample, it allowed for the liquefaction for 45 minutes ands the liquefaction time is find out. Color of the specimen is observed. Volume of total semen ejaculation is measured. $P^H$ of the semen was checked by use of the $P^H$ paper. The parameters were compared with normal semen parameters to find the abnormalities in the sperm.

**Sperm concentration**

10µl of thoroughly mixed semen is placed on a Makler counting chamber and the cover glass is placed and observed under the microscope. the total number of sperms in vertical 10 squares was counted in order to find the count and slide viewed under 40 x objective to assess motility (rapid, progressive, moderate and sluggish). Immotile WBC, RBC, epithelial cells and agglutination of sperms, the 3 to 4 columns were countered and the average of it is taken and this gives the concentration in millions per ml. These microscopic parameters were compared with the normal semen parameters. These parameters used to decide the infection of microorganisms in sperms.

**Calculate the percentage of motile and immotile sperm**

Spermatozoa motile immotile is estimated by the microscopic examination using Makler chamber. This concentration gives the values per cubic mm counted motile, immotile sperms invertical 10 squares were subjected the formula and the percentage of motility and total sperm count is found out.

$$\text{Motility percent} = \frac{\text{Number of motile sperm}}{\text{Total number of sperm}} \times 100$$

Where,

Total number of sperm=Motile + Dead

Number motile sperm = Number of motile sperms x volume

When the volume of the sperm sample is below 1ml, the total sperm count is not taken into account.

**Morphology**

Morphology of the sperm exposes the quality of individual sperm. Good quality sperm is needed for the fertilization of the oocyte.

**Smear preparation**

Semen sample were allowed to liquefy at 37°C for 30 min. The liquefied sample was pelleted at 250gm for 10min and resupended in its own supernatant to an approximate concentration of 2 x $10^8$

cells/ml. Thin smear were prepared on precleaned deflated slides and then air dried for 30-60 min. Dried smear were fixed with glutaraldehyde for 30min-12 hrs and air dried.

## Staining methods

Coplin jar prior to staining procedure was followed. The air dried slides were analyzed under phase contrast microscope under 40 x objectives. The sperm stained with toluidine blue appear from light blue to dark purple in colors.

$$\% \text{ of DNA damage} = \frac{\text{Number of sperm defect in DNA}}{\text{Total number of defect sperm}} \times 100$$

Where,

Total number of defect sperm = No of DNA defect + No of acrosome defect.

## Specimen preparation for cryopreservation

Neat semen or prepared sperm (washed) may be cryopreserved. Processing the sperm and removing the Seminal fluid and microbial infection prior to cryopreservation offers the possibility to remove and save the sperms from microbial infection, which is helpful to concentrating count, motile sperm. After thawing, Cryopreserved specimen from microbial infected infertile couple, which keeps the specimen enough to ART procedure without drastic twon, Moreover, sperm preparation prior to cryopservation provides "physicians with an "IUI, IVF, ICSI specimen after thawing. Sperm preparation may be accomplished using a colloidal suspension gradients, the sperm preparation ttechnique are divided into two types.

## Swim-up Technique

The swim up technique is the earliest and most widely used technique of sperm separation from non motile sperm and cellular debris. After liqufaction the ejaculate is washed 1:5 with culture medium (37°C). After 10 minutes of centrifugation (300-400g). The sample is loosened and gently overlaid with 0.5-1 ml of medium. The sample is stored inside an incubator (37°C) for 30 mins to allow the motile sperm to swim-up into the overlying medium. The supernatant is removed and 0.3 – 0.5 ml of it is used for insemination.

## Swim-Down Technique

The density gradient centrifugation has been used in the past mainly for the improvement of poor quality semen samples. Approximately 2ml of 80% percoll at the bottom of a 10ml tube overlaid by 2ml of 80% percoll. The semen sample is layered on top of the column which is then centrifuged at 600g for 20min.

After centrifugation 0.5-0.7 ml of the so-called soft pellet from the bottom of the tube is aspirated resuspended in 1-2ml of medium and centrifuged for 5-10 minutes at 200g to wash out most of the percoll. The pellet is resuspended in 0.5ml of medium and then used for the IUI procedure.

# Result and Discussion

Semen samples obtained from 30 patients were assessed for normal semen parameters and analyzed together for sperm DNA integrity by TB staining (Tables 1, 2). Count, motility, morphology and DNA integrity

assessment were taken into account and compared for damaged DNA.

Out of 30 patients, the results obtained were compared with Count and DNA damage, Motility and DNA damage, Count, motility and DNA damage. from the above studies, the results are that, Out of 30 patients, 6 have < than 20million/ml and the % of DNA damage is 10% and Out of 30 patients, 24 have > than 20million/ml and the % of DNA damage is 20%. Out of 30 patients, 10 has < than 50% motility and the % of DNA damage is < 30% for 10 patients. Whereas the other 20 patients, the motility is > than 50% but DNA damage is than 30%.

If the count and motility is affected, there is a significant reduction of normal sperm parameters and the % of DNA damage is higher. The damage to human sperm DNA might adversely affect reproductive outcomes and that the spermatozoa of infertile men possess substantially more sperm DNA damage than do the spermatozoa of fertile men. There is now some evidence to suggest that markers of sperm DNA integrity may be better measures of male fertility potential than conventional measures.

Conventional semen parameters fall shock. The study analyzed that most of the patients who have DNA damage > 30% have less chance for fertility potential. It is advisable for the patients who have > than 30% DNA damage either to undergo IVF or ICSI treatments. This study insists that DNA integrity as a screening test along with conventional semen analysis. This units the chance of patients undergoing IVF treatments, to limit the number of IUI cycles and to directly counsel from the ICSI or IVF. Still wide research is needed to enhance sperm DNA integrity assessment.

The cryopreserved samples can be stored for years together in cryostorage tank under liquid nitrogen. DNA damage of individual samples can be prevented to some extent in cryopreserved samples gives good normal motile sperm for ARTs procedure. Cryopreservation is a useful technique which helps an individual who has Ejaculatory dysfunction, The man, who has low count and motility, The man, who undergoes chemotherapy, The man, who travel abroad, The man, who undergoes testicular biopsy.

Cryopreservation has been reported to cause changes in sperm morphology including damage to mitochondria, the acrosome and the sperm tail. Sperm motility is particularly sensitive to such damage (Henry *et al.,* 1993). Despite many advance in cryobiology the salvage rate has changed little (Centola *et al.,* 1992; Sharma and Agarwal, 1996). Cryopreservation of human spermatozoa is used extensively in artificial insemination and IVF programs. A freezing semen sample, either raw or extended, has not effect on the extent of DNA denaturation. However, DNA samples from fertile men were more resistant to freezing damage than those of infertile men, especially if they were frozen in the presence the seminal plasma, which affords antioxidant protection (Donnellay *e. al.,* 2001).

The protection of DNA has important implications in the use of freeze-thawed donar spermatozoa for insemination. This is extreme relevant for ozoospermia who may have spermatozoa banked for long-term storage prior to chemotherapy or radiotherapy. It is critical to optimize the protocols that are used to prepare and freeze sperm samples from infertile men to protect their DNA.

**Table.1** Criteria of the semen samples

| S. No | Sample | Age | Count million/ ml | Motility in % | Abstinence | Colour | Reaction | Viscosity | WBC million | Liquification | Volume ml | pH | TSC million | Opinions |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Sample - 1 | 28 | 31 | 77 | 0 days | go | alkaline | thin | 0.4 | normal | 1.5 | 7.6 | 46.5 | normozoospermia |
| 2 | Sample – 2 | 28 | 90 | 67 | 8 days | go | alkaline | thin | 0.4 | normal | 6.0 | 7.4 | 90 | normozoospermia |
| 3 | Sample – 3 | 28 | 27 | 88 | 5 days | go | alkaline | thin | <1 | normal | 4.0 | 7.6 | 108 | normozoospermia |
| 4 | Sample – 4 | 29 | 181 | 62 | nil | go | alkaline | viscous | 0.6 | after 2 hrs. | 1.8 | 7.6 | 326 | normozoospermia |
| 5 | Sample – 5 | 29 | 190 | 86 | 8 days | go | alkaline | thin | 0.6 | normal | 3.6 | 7.8 | 684 | normozoospermia |
| 6 | Sample – 6 | 29 | 67 | 25 | 9 days | go | alkaline | thin | 1 | normal | 2.0 | 7.6 | 134 | normozoospermia |
| 7 | Sample – 7 | 31 | 98 | 41 | 3 days | go | alkaline | viscous | 0.5 | normal | 2.0 | 7.8 | 196 | normozoospermia |
| 8 | Sample – 8 | 32 | 12 | 42 | 3 days | go | alkaline | thin | >1 | normal | 3.0 | 7.8 | 36 | normozoospermia zoospermia |
| 9 | Sample – 9 | 33 | 21 | 57 | 5 days | go | alkaline | thin | 0.4 | normal | 0.8 | 7.6 | 21 | normozoospermia |
| 10 | Sample – 10 | 33 | 100 | 80 | nil | go | alkaline | thin | 0.6 | normal | 1.5 | 7.8 | 150 | normozoospermia |
| 11 | Sample – 11 | 33 | 98 | 91 | 4 days | go | alkaline | thin | 0.6 | normal | 2.5 | 7.8 | 245 | normozoospermia |
| 12 | Sample – 12 | 33 | 38 | 52 | nil | go | alkaline | thin | <1 | normal | 1.0 | 7.6 | 38 | normozoospermia |
| 13 | Sample – 13 | 33 | 10 | 60 | nil | go | alkaline | thin | 0.6 | normal | 5 | 7.8 | 50 | mild asthenozoospermia |
| 14 | Sample – 14 | 33 | 72 | 10 | 10 days | go | alkaline | viscous | 0.9 | normal | 3.5 | 7.8 | 252 | astheno azoospermia |
| 15 | Sample – 15 | 34 | 51 | 59 | 20 days | go | alkaline | thin | 0.6 | normal | 7.0 | 7.6 | 357 | normozoospermia |

| S. No | Sample | Age | Count million/ ml | Motility in % | Abstinence | Colour | Reaction | Viscosity | WBC million | Liquification | Volume ml | pH | TSC million | Opinions |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | Sample – 16 | 35 | 18 | 56 | 3 days | go | alkaline | thin | 0.4 | normal | 3.2 | 7.4 | 57 | oligo asthenozoospermia |
| 17 | Sample – 17 | 35 | 94 | 57 | 7 days | go | alkaline | thin | 0.6 | normal | 2.2 | 7.8 | 207 | mild |
| 18 | Sample – 18 | 35 | 103 | 87 | 6 days | go | alkaline | thin | 0.6 | normal | 0.5 | 7.6 | 103 | normozoospermia |
| 19 | Sample – 19 | 36 | 15 | 80 | 20 days | gy | alkaline | thin | 0.4 | normal | 1.8 | 7.2 | 27 | oligo asthenozoospermia |
| 20 | Sample – 20 | 36 | 48 | 62 | 1 days | go | alkaline | thin | 0.6 | normal | 0.8 | 7.8 | 48 | normozoospermia |
| 21 | Sample – 21 | 36 | 68 | 85 | 2 days | go | alkaline | thin | 0.5 | normal | 1.0 | 7.8 | 68 | normozoospermia |
| 22 | Sample – 22 | 37 | 30 | 80 | nil | go | alkaline | thin | 0.6 | normal | 1.5 | 7.6 | 45 | normozoospermia |
| 23 | Sample – 23 | 37 | 54 | 54 | nil | go | alkaline | thin | <1 | normal | 3.4 | 7.6 | 184 | mild asthenozoospermia |
| 24 | Sample – 24 | 37 | 68 | 23 | 4 days | go | alkaline | thin | 0.5 | normal | 3.5 | 7.6 | 238 | asthenozoospermia |
| 25 | Sample – 25 | 38 | 67 | 69 | 5 days | go | alkaline | thin | 0.6 | normal | 0.4 | 7.6 | 67 | normozoospermia |
| 26 | Sample – 26 | 38 | 11 | 27 | 3 days | go | alkaline | viscous | 0.6 | after 2 hrs | 1.5 | 7.6 | 16.5 | oligo asthenozoospermia |
| 27 | Sample – 27 | 39 | 20 | 35 | 15 days | go | alkaline | thi | 0.5 | normal | 6.5 | 7.8 | 130 | asthenozoospermia |
| 28 | Sample – 28 | 41 | 14 | 14 | 4 days | go | alkaline | viscous | 0.4 | after 1.30 hrs | 3.0 | 7.6 | 42 | oligo asthenozoospermia |
| 29 | Sample – 29 | 42 | 59 | 32 | 10 days | go | alkaline | thin | 0.6 | normal | 2.0 | 7.6 | 118 | asthenozoospermia |
| 30 | Sample – 30 | 42 | 23 | 60 | 7 days | go | alkaline | thin | <1 | normal | 3.0 | 7.6 | 69 | mild asthenozoospermia |

GO – Grey opalscent; GY – Greyish Yellow; TSC – Total Sperm Count

Table.2 Assessment of Sperm DNA integrity by tolutidine blue.

| S.No. | Name | Age | Normal | DNA defect | No acrosome | % DNA defect |
|---|---|---|---|---|---|---|
| 1 | Sample - 1 | 28 | 48 | 33 | 19 | 17.16 |
| 2 | Sample – 2 | 28 | 35 | 45 | 20 | 29.25 |
| 3 | Sample – 3 | 28 | 37 | 48 | 15 | 30.24 |
| 4 | Sample – 4 | 29 | 56 | 32 | 12 | 14.08 |
| 5 | Sample – 5 | 29 | 60 | 26 | 14 | 10.40 |
| 6 | Sample – 6 | 29 | 36 | 52 | 12 | 33.28 |
| 7 | Sample – 7 | 31 | 37 | 45 | 18 | 28.35 |
| 8 | Sample – 8 | 32 | 30 | 50 | 20 | 35.00 |
| 9 | Sample – 9 | 33 | 44 | 39 | 17 | 21.84 |
| 10 | Sample – 10 | 33 | 48 | 38 | 14 | 19.76 |
| 11 | Sample – 11 | 33 | 35 | 40 | 25 | 32.00 |
| 12 | Sample – 12 | 33 | 37 | 46 | 17 | 28.98 |
| 13 | Sample – 13 | 33 | 37 | 35 | 28 | 22.05 |
| 14 | Sample – 14 | 33 | 33 | 46 | 21 | 30.81 |
| 15 | Sample – 15 | 34 | 19 | 30 | 51 | 24.30 |
| 16 | Sample – 16 | 35 | 36 | 40 | 24 | 25.60 |
| 17 | Sample – 17 | 35 | 34 | 42 | 24 | 27.72 |
| 18 | Sample – 18 | 35 | 34 | 45 | 21 | 31.28 |
| 19 | Sample – 19 | 36 | 30 | 41 | 29 | 28.70 |
| 20 | Sample – 20 | 36 | 44 | 44 | 12 | 24.69 |
| 21 | Sample – 21 | 36 | 41 | 43 | 16 | 25.37 |
| 22 | Sample – 22 | 37 | 33 | 54 | 13 | 36.18 |
| 23 | Sample – 23 | 37 | 42 | 48 | 10 | 27.80 |
| 24 | Sample – 24 | 37 | 20 | 45 | 35 | 36.00 |
| 25 | Sample – 25 | 38 | 52 | 30 | 18 | 14.40 |
| 26 | Sample – 26 | 38 | 34 | 43 | 23 | 28.30 |
| 27 | Sample – 27 | 39 | 50 | 30 | 20 | 15.00 |
| 28 | Sample – 28 | 41 | 31 | 45 | 24 | 31.03 |
| 29 | Sample – 29 | 42 | 30 | 46 | 24 | 32.20 |
| 30 | Sample – 30 | 42 | 36 | 43 | 19 | 28.80 |

In support of the beneficial role of the sperm preparation protocols, a general improvement in nuclear maturity may be seen in post-swim-up samples (Sanchez *et al.,* 1996)

Numerous studies have examined the possible influence of sperm DNA integrity on reproductive outcomes after both standard IVF and IVF/ICSI. There is not consistent relation between sperm DNA damage and fertilization rates during IVF or IVF/ICSI (Lopes *et al.,* 1998, Tomlinson *et al.,* 2001). Neither fertilization nor early embryo development is dependent on sperm DNA integrity, since the embryonic genome is not expressed until after the second cleavage division (i.e., at the 4-cell embryo). There is also no consistent relation between sperm DNA damage and embryo quality after ICSI (roughly half of the studies have shown an adverse effect of DNA damage on embryo quality). However, high levels of sperm DNA damage are inversely related to pregnancy rates in most, but not all, studies (Larson-ccok *et al.,* 2003, Seli *et al.,* 2004, Zini *et al.,* 2005). These clinical observations are not entirely consistent with animal studies, which indicate that DNA damage is associated with both poor embryo development and poor pregnancy outcomes. This is probably because the stringent process of sperm and embryo selection during ICSI will mitigate the potential adverse effects of sperm DNA damage on reproductive outcomes.

The current understanding is that sperm chromatin is tightly packaged by protamines, but up to 15% of the DNA remain packaged by histones at specific DNA sequences (i.e., there is a nonrandom association between histones and DNA sequences). The histone-bound DNA sequences are less tightly compacted, and it is thought that these DNA sequences or genes may be involved in fertilization and early embryo development (Gatewood *et al*., 1987). The retained histones are associated with the nuclear periphery and with telomeres (Gineitis *et al.,* 2000). Infertile men, as compared with fertile controls, have an increased sperm histone: protamine ration (Steger *et al.*, 2000, Oliva *et al.,* 2006). An excess of nuclear histones (> 15%) results in poorer chromatin compaction and a subsequent increased susceptibility to external stresses (e.g. oxidation or temperature elevation in the female reproductive tract) (Kosower *et al.*, 1992).

## References

Ahmadi, A., and Ng, S.C.1999. Fertilizing ability of DNA-damaged spermatozoa, J. Exp. Zool. 284: 696-704.

Anderson, S., A.T. Bankier and Barrel,l B.G. 1981. sequence and organization of the human motochondrial genome. Nature. 290: 457-65

Aravindan, G.R., J.Bjordah and Jost, L.K.1997. Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. Exp .Cell. Res. 236: 231-7

Bianchi, P.G., G.C. Manicardi and Bizzaro, D.1993. Effect of deoxyribonucleic acid protamination of fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. Biol. Reprod. 49:1083-8.

Brewer,L.R., M. Corzett and Bathorn,R. 1999. Protamine induced condensation and decondensation of the same DNA molecule. Science. 286: 120-3

Carrell, D.T and Liu, L.2001. Altered

protamine to expressions is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. J. Androl.22: 604-10.

Centola, G.M., R.F. Raubertas and Mattox, J.H.1992. Cryopreservation of human semen. Comparison of cryopreservation, sources of variability and prediction of post thaw survival. J.Androl.13:283-288.

Cho,C., H. Jung-Ha and Willis, W.D. 2003. Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. Biol. Reprod. 69: 211-7.

Darzynkiewics, Z., and Kapusinski, J. 1990. Acridine orange : a versatile probe of nucleic acids and other cell constituents. In Melamed MR, Lindmo T, and Mendelsohn ML (eds), Flow Cytometry and Sorting, 2nd edn, Wiley-Liss, inc, New York .pp.291-314.

Donnelly, E., N. McClure and Lewis, S.E. 2001. Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA ingrity. Fertility.Sterility. 76: 892-900.

Erenpreisa, J., J. Bars and Lipatnikova, V. 2001. comparative study of cytochemical tests for sperm chromatin integrity. J. Androl. 22:45-53.

Erenpreisa, J., T. Freivalds and Selivanova, G.1992. influences of chromatin condensation on the adsorption spectra of nuclei stained with tolutidine blue. Acta. Morphol. Hung. 40: 3-10.

Evenson, D.P., L.K. Jost, D. Marshall, M.J. Zinaman, E. Clegg, K. Purvis, P. De Angelis and Claussen, O.P.1999. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Human Reproductive. 14: 1039-1049.

Gatewood, J.M., G.R. Cook and Balhorn, R. 1987. Sequence – specific packaging of DNA in human sperm chromatin. Science. 236: 962-964.

Gineitis, A.A., I.A. Zalenskaya and Yau, P.M. 2000. Human sperm telomere-binding complex involves histone H2B and secures telomere membrane attachment .J. Cell Biol. 151 : 1591-8.

Henry, M.A., E.E. Noiles, P.Gaod, Mazur and Crister .J.K.1993. Cryopreservation of human spermatozoa. the effects of colling rate and warming rate on the maintenance of motility, plasma membrane integrity, and mitochondrial function. Fertility Sterility. 60: 911-918.

Kao, S.H., H.T. Chao and Wei, Y.H. 1998. Multiple deletion of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa, *Mol* Human Reproductive. 4: 657-66.

Kodama, H., R. Yamaguchi and Fukuda, J.1997. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. Fertility Sterility. 68: 519-24.

Kosower, N.S., H. Katayose and Yanagimachi, R. 1992. thiol-disulfide status and acridine orange fluorescence of mammalian sperm nuclei. J. Androl. 13:342-8.

Larson-Cook, K.L., J.D. Brannian, K.A. Hensen, K.M. Kasperson, E.T. Aamold and Evenson, D.P. 2003. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. Fertility Sterility. 80: 859-902.

Loft, S., T. Kold-Jensen, N.H. Hjollund,

A. Giwercman, J. Gyllemborg and Ernst, E. 2003. Oxidative DNA damage in human sperm influences time to pregnancy. Human Reproductive. 18: 1265-72.

Lopes,S., J.G. Sun, A. Jurisicova, J. Meriano and Casper, R.F. 1998. Sperm deoxyribonucleic and fragmentation is increased in poor-quality semen and correlates with failed fertilization in intracytoplasmic sperm injection, Fertility. Sterility. 69: 528-32.

Oliva,R., 2006. Protamines and male infertility. Human Reproductive Update. 12: 417-35.

Polge, C., A.U. Smith and Parkes, A.S. Revivial of spermatozoa after vitrification and dehydration at low temperature. Nature. 169: 626-627.

Sanchez, R., T. Stalf, O. Khanaga, H. Turley, H. Gips and Schill, W.B.1996. Specrm selection methods for intracytoplasmic sperm injection in andropgical patients .A, Assist. Reprod. Genet. 13: 228-33.

Seli, E., D.K. Gardner and Schoolcraft, W.B.2004. Extent of nuclear DNA damage in ejaculated psermatozoa impacts on blastocyst development after *in vitro* fertilization, Fertility. Sterility. 82: 378-83.

Sharma, R.K., and Agarwal, A. 1996. Sperm quality improvement in cryopreserved Human Semen .J.Urol. 156 : 1008-1012.

Spano, M., J.P. Bonde and Hjollund, H.I.2000. Sperm chromatin damage impairs human fertility. Fertility. Sterility. 73: 43-50

Steger, K., K. Pauls and Klonisch, T. 2000. Expression of protamine-1 and -2 mrna during human spermiogenesis. Mol. Human. Reproductive. 6: 219-25.

Sylven, B., 1954. Metachromatic dye-substrate interactions. Q.J.Microsc. Sci. 95: 95-114.

Tomlinson, M.J., O. Moffatt and Manicardi. G.C.2001. Interrelationship between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation : implications for assisted conception. Human Reproductive. 16:2160-5.

Tournaye, H., 1999. Surgical sperm recovery for intracytoplasmic sperm injection. Which method is to be preferred? Human. Reproductive. 14: 71-81.

Ward, W.S., and Coffey, D.S.1991. DNA packaging and organization in mammalina spermatozoa; comparison with somatic cells. Biol. Reprod. 44: 569-74.

World Health Organization (WHO)., 1999. Laboratory Manual for the examination of human semen and sperm, Cambridge, United Kingdom: *Cambrdige University Press.*

Zini, A., R. Bielecki and Phange, D. 2001. Correleations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertilemen, Fertility. Sterility. 75: 674-7.

Zini, A., J. Meriano and Kader, K. 2005. Potential adverse effect of sperm DNA damage on embryo quality after ICSI. Human .Reproductive.20: 476-80.