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A Study of Hypo Osmotic Swelling Test in Human Spermatozoa

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A B S T R A C T

A study in which couples having a male partner with HOS Test scores <50% would be randomized to conventional IVF. IVF is an expensive and potentially risk procedure and conventional IVF without ICSI is an ineffective therapy. Only viable sperm cells, with chemically and physically intact membrane, undergo tail swelling due to water influx under Hypo Osmotic condition a higher fertilization rate selected by the HOST and the studies of HOS test is able to predict pregnancy rate and outcome in couples undergoing in vitro fertilization / intra cytoplasmic sperm injection and intrauterine insemination procedures. Our results are in agreement with those of other authors reporting a threshold value of 60% overall sperm swelling rate as an indicator of normal fertility potential of human spermatozoa. This HOS Test is a promising method of identifying the live and dead spermatozoa for IVF/ICSI and IUI. The HOS Test is not included in standard semen analysis and is considered with the workup of unexplained infertility. The hypo-osmotic swelling (HOS) test evaluates the functional integrity of the sperm's plasma membrane and also serves as a useful indicator of fertility potential of sperm. The HOS test predicts membrane integrity by determining the ability of the sperm membrane to maintain equilibrium between the sperm cell and its environment.

Introduction

The hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test (HOST/HOS test) for investigating the functional integrity of the human sperm membrane has been introduced as a useful assay in the diagnosis of the infertile semen by *Jeyendran et al. (1984)*. The principle of the HOS assay is based on fluid transport across the sperm tail membrane under hypo-osmotic conditions until equilibrium is reached. Due to this influx of fluid, the tail

expands and bulges in characteristic patterns, considered as hypo-osmotic response, which can readily be identified with a phase-contrast microscope (*Takahashi et al., 1990; Mladenovic et al., 1995)*. The rationale of the test is based on the assumption that an undamaged sperm tail membrane permits passage of fluid into the cytoplasmic space making such space swollen, and the pressure generated makes tail fibres curl, while the damaged or chemically inactive tail membrane allows fluid to pass across the membrane without any accumulation and accordingly no cytoplasmic swelling and curling of the tail occur (*Mordel et al., 1993; Mladennovic et al., 1995)*. The resultant swelling of the tail thus means an intact membrane and presumably normally functioning spermatozoa *(Jeyendran et al., 1984, 1992)*. The advantage of the HOST is that it is very simple and repeatable (World Health Organization (WHO), 1992). In fact, the HOS assay is the simplest test of all the WHO recommended sperm function tests, and thus is widely used (*Jeyendran et al., 1992; Mladenovic et al., 1995*).

Most recently the HOST has been recommended for use in discriminating viable from nonviable spermatozoa for intracytoplasmic sperm injection (ICSI) in cases of complete asthenozoospermia (*Desmet et al., 1994; Casper et al., 1996; Verheyen et al., 1997)*. Because of its simplicity, this procedure has been found to be of benefit in testicular biopsies and in ejaculated spermatozoa lacking motility (*Ahmadi and Ng, 1997; Barros et al., 1997; Liu et al., 1997)*. Although most of the investigations on HOST showed good predictive power, some did raise concern about its validity (*Chan et al., 1985; Fuse et al., 1991; Jager et al., 1991*). To date, there is no single test that would reliably predict the fertilizing capacity of human semen (*Vantman et al., 1987; Mordel et al., 1993)*. Similar to other sperm function tests, the HOST in its present form does not provide unequivocal information regarding the fertilizing ability of the spermatozoa (*Mordel et al., 1993; Check et al., 1995; Kiefer et al., 1996)*. From a recent review of the HOST related research it appears that much of the confusion is due to the interpretation of the data rather than to the accuracy of the HOS results (*Jeyendran et al., 1992*).

Infertility or subfertility is defined as a condition in which a couple will not achieve a pregnancy within one year. Subfertility generally describes any form of reduced fertility with a prolonged time of non-conception. Infertility may be used synonymously with sterility, with only sporadically occurring spontaneous pregnancies. The prevalence of infertility in Finland is thought to be approximately 15%, which means that every sixth couple will suffer from infertility in some part of their potentially fertile years (*Tiitinen 2008*). Even though infertility has no effect on physical health, it influences the mental health and social life of infertile couples. Approximately 30% of cases of infertility are the result of female factors, 30% are connected with male factors, 30% are connected with both male and female factors and in 10–20% of cases the reason for infertility remains unclear. Of infertile couples, 22% are regarded as sterile. Disorders cause infertility in 15–30% of infertile couples and – additionally – they also have an impact on the choice of treatment.

The determination of sperm viability in semen displaying a low percentage of motile cells is useful to indicate whether spermatozoa may be revived after transfer from semen to medium and thus be of

therapeutic use. Most routine methods for measuring sperm viability involve examining the permeability of the cell membranes: those of the tail in the case of the hypo-osmotic swelling test, and those of the head when impairment dyes such is eosin nigrosin stain.

Assessing of sperm vitality when motility is reduced or completely absent, and thus cannot be of any help in assessing vitality, eosin-Y allows the detection of living sperm cells. This procedure is used for diagnostic purposes only, as dyed vital spermatozoa cannot afterwards be used for microinjection. The HOS test is used only when no motile spermatozoa are found. The test can be used for diagnostic purposes but has a major advantage over the eosin-Y test: the analyzed sperm cells can be used for microinjection. This means that vital spermatozoa for microinjection can be selected from an immotile sample. A normal semen sample should not contain more than 106 leucocytes/ ml. An increased number of leucocytes in the ejaculate (leucospermia) indicate an infection in the genital tract. As the presence of leucocytes affects sperm values, in our center, microscopic leucocytes identification is always confirmed with a detection kit. The number of immature germ cells should be ≤106/ ml. It is extremely important to identify immature germ cells in patients with a low number of sperms or with azoospermia to determine precisely the extent of the sperm production defect. In our center, we use Bryan-Leishman staining and Test-simplest kit containing pre-dyed slides to detect immature germ cells.

Spermatozoa morphology is a very good indicator of sperm quality. In our center, spermatozoa morphology assessment is done according to criteria in the "WHO Manual for Examination and Processing of Human Sperm, 5th edition" using "Sperma stain", which stains the nucleus red and the acrosome, neck and tail green. After staining, spermatozoa morphology is analyzed under a microscope at 100X magnification. Both abnormal and normal forms are recorded and over 200 spermatozoa are evaluated. Thirty-eight distinct types of anomaly in the head, neck and tail of the sperm cell, can be detected. If the ratio of spermatozoa with a normal morphology in a sample is below 4%, this condition is diagnosed as teratozoospermia. Although rates below or above 4% are considered to have some significance with regard to fertilization and pregnancy, every laboratory has to determine its own thresholds. For example, according to Kruger strict criteria (1993), if this value is below 4%, the sub-distribution of anomalies is scrutinized: head anomalies may be severe or mild, affecting fertilization differently.

Studies have established a relationship between sperm morphology, fertility, embryo development and pregnancy outcomes. Sperm morphology analysis is not only important for the evaluation of male fertility but also for the decision regarding which ART strategy should be adopted. Severe morphological anomalies affect the fertilization capacity of the sperm cell. The most important anomalies are: macrocephaly (or megalohead), globozoospermia (or roundhead), and tail-stump. If the majority of spermatozoa are morphologically abnormal, the best-morphology spermatozoa are selected from among the whole sample. However, the treatment may nevertheless result in a low fertilization rate, slow-growing embryos and poor quality embryos.

In laboratory, if the normal sperm rate is below the expected value or if severe male infertility is suspected, as well as the routine semen analysis and morphology evaluation, semen is also examined by Motile Sperm

Organelle Morphology Examination (MSOME), a technique using a high magnification microscope. MSOME is also used for couples with repeated ART failures and/ or with unexplained infertility. The advantage of the MSOME technique is its magnification power (8050X). The major difference between the initial semen analysis and MSOME is the sperm preparation technique. For standard sperm morphology evaluation, sperm is fixed onto the microscope slide and stained with special dyes; therefore motile and immotile spermatozoa are all assessed together. However, for MSOME, only motile spermatozoa are analyzed, giving a better indication of the quality of the sperm that will be selected for microinjection on oocyte pick-up (OPU) day.

When sperm is subjected to MSOME, various parameters are investigated: spermatozoa with a normal mid-piece and tail are preselected, and the presence of vacuoles in the head is scrutinized. Accordingly, the sperm is graded from 1 to 4, with 1 being spermatozoa with normal morphology and no vacuoles and 4, spermatozoa with an abnormal head and/ or numerous vacuoles. All versions of the World Health Organization (1980,1987, 1992, 1999) Handbook for the Examination of Human Semen and Semen-Cervical Mucus Interaction have presented 2 tests of sperm viability, involving examination of either wet preparations or air dried smears of semen to which eosin has been added. For the wet preparations the World Health Organization (1980) suggested the use of a negative-phase objective with which dead spermatozoa appeared yellow and live spermatozoa bluish, contrasting with bright-field (non phase) optics in which dead cells were red and live cells white. The World Health Organization (1987, 1992) suggested the use of either bright-field or phase-contrast optics for the wet preparation, whereas the World Health Organization (1999) recommended bright-field optics for wet preparations and negative-phase for smears of eosin-only wet preparations. A recent critical analysis of the alternative method, eosin-nigrosin smears, concluded that a modified 1-step method using isotonic media should be incorporated into the laboratory routine (*Bjorndahl et al.,2004*). Semen analysis is routinely used to evaluate the male partner in infertile couples and to assess the reproductive toxicity of environmental or therapeutic agents. Although widely used thresholds for normal semen measurements have been published by the World Health Organization (WHO), the available norms for sperm concentration, motility, and morphology fail to meet rigorous clinical, technical, and statistical standards. In recognition of these limitations, the nomenclature in the most recent WHO manual for semen evaluation was changed from "normal" to "reference" values. Two recent prospective studies of semen quality and fertility concluded that the current WHO reference values should be reconsidered.

Freezing procedure is just as important as the freezing procedure in terms of its impact on the survival of spermatozoa. The modification of the temperature curve involved in freezing and thawing of semen inevitably reduces the proportion of motile spermatozoa and causes ultra structural, biochemical and functional damages. For this reason, various researches have been carried out about the most ideal thawing technique of frozen semen. Because of the high surface-to-volume ratio, the straws are significantly sensitive to temperature changes. The post thaw fertilizing ability of spermatozoa is greatly affected by protocols such as thawing temperature and duration. Diluents types, freezing rate, glycerol levels

and glycerol equilibration time interact with the above thawing protocols. Evaluation of the quality of frozen semen has been based on a variety of methods, including routine semen analysis (motility, morphology and acrosome integrity), zonafree hamster ova test and hypo-osmotic swelling test. The plasma membrane is of crucial importance to freeze-thaw survival of spermatozoa and regarded as the primary site of freezing injury.

Spermatozoa may be cryopreserved to maintain their viability and thawed for use in a future ART attempt. The first successful birth from a cryopreserved sperm sample occurred in 1973. Intrauterine Insemination (IUI) is a fertility procedure in which sperm are washed, concentrated and injected directly into the women's uterus. In natural intercourse, only a fraction of sperm makes it into the fallopian tubes where fertilization takes place. IUI aims to increase this number. Research studies have shown that IUI is most successful when coupled with fertility drugs to enhance ovulation. This technique is often called stimulated IUI. IUI is usually recommended for couples with no known cause of infertility who have been trying to conceive for at least one year. The benefit of IUI is achieving a pregnancy.

The simplified one-step technique, exposing sperm to a mixture of eosin and nigrosin, was introduced on boar, bull and ram sperm (*Campbell et al., 1956*), on rabbit sperm (*Beatty,1957)* and further evaluated for various mammalian sperm(*Dott and Foster, 1972*). The technique was used on washed human sperm by Mortimer (*Mortimer et al., 1990*). Although Mortimer pioneered its use on human sperm in semen *(Mortimer, 1985; 1994),* no formal evaluation on its use on sperm in raw semen has been published. Since the one-step technique is now widely in use for basic semen analysis, it is vital to the ongoing international standardization and quality improvement (*BjoErndahl et al., 2002*) to evaluate the technique on sperm in semen.

The conventional semen analysis represented by motility largely depends on energy production originating sperm cell concentration, motility, morphology and vitality from the mitochondrial compartment of the sperm are still used for predicting the fertility of sperm in most of mid-piece. Therefore, loss of motility is not only related animal and human insemination laboratories. The functional are poorly correlated with fertility .In cases in which tests which assess membrane function and integrity sperm motility is good, live/dead stains typically provide may be more accurate predictor of *in vitro* or *in vivo* little additional information, since sperm motility is highly fertilization than other methods use.

Studies have been performed to evaluate the correlation of subnormal hypo-osmotic swelling test (HOST) scores with other semen parameters that are believed to be predictive of fertilization potential, albeit with various conclusion. Probably the best way to determine if a test correlates with oocyte fertilization potential is to compare fertilization rates following in vitro fertilization (IVF) with sperm with subnormal vs normal HOST scores. A previous in vivo study found a pregnancy rate of 83% in an 8-month treatment period in infertile couples whose male partner had HOST scores of 50% compared with no pregnancies in couples in whom the male partner's HOST score was 50% even if all semen parameters were normal (*Check et al, 1989*).

The only study that might be interpreted to mean that low HOST scores may not be

associated with poor pregnancy rates was our own publication of frozen embryo transfers. Both pregnancy rates per patients and per cycle was significantly higher (P > .05) in group1 than in group 2. The HOS test may be considered an easy and reliable test in identifying among subfertile men those who have a greater possibility of causing pregnancy.

The aim of this study to explore the unexplained male infertility and to find out the functional disabilities of the sperm regarding the fertilizing potential.

The objectives of the study are IUI and IVF for Asymptomatic and Symptomatic subfertile patient group, Hypo Osmotic Swelling Test (HOS TEST), Eosin – Nigrosin Staining (Vitality staining), Leukocytes staining (White blood cell staining) and Semen Microscopic Parameters and Macroscopic Parameters study.

Materials and Methods

Patients visiting the infertility clinic of NOVA IVF Fertility center in Chennai, evaluation and treatment participated in this prospective study. A total of 40 subjects were included in the present study, after provision of a written, informed consent. All subjects were asked to provide semen samples after 3-5 days of ejaculatory abstinence. Semen specimens were produced by masturbation directly into a sterile plastic container, in a room specially provided for this purpose and located adjacent to the laboratory.

Patients ranging 20 to > 40 were identified from the regional andrology lab. The method is routine semen analysis. The patients were divided into 2 groups normal and subnormal. Both pregnancy rates per patients and per cycle was significantly higher (P <.05) in group 1 than in group 2 or group 2 than in group 1.The Hypo Osmotic Swelling Test (HOS test) may be considered an easy and reliable test in identifying among subfertile men those who have a greater possibility of causing pregnancy.

Hos test

The hypo-osmotic swelling test (HOST/HOS test) first described by (*Jeyendran et al., 1984*), this test evaluates the functional integrity of the sperm membrane (*Liu et al., 1997*). The World Health Organization recommends to use the hypo-osmotic swelling test as vitality test alternative to dye exclusion. HOST is useful when staining of spermatozoa is avoided (World Health Organization, 2010). Although the percentage of sperm survival and motility may be affected by exposure to hypo-osmotic solutions, this does not necessarily imply a decreased functionality of the surviving sperm in ICSI (*Verheyen et al., 1997*). The original HOST solution consists of a mixture of equal parts of fructose and sodium citrate with a calculated ionic strength of 0.15. HOST solution, a maximal number of clearly swollen spermatozoa is identifiable (*Jeyendran etal., 1984*). Live spermatozoa with normal membrane function it show swelling of the cytoplasm and curling of the tail due to water influx when exposed to hypo-osmotic conditions. These changes are visualized easily under light microscopy.

HOS Reagent is used to identify the live immotile spermatozoa in 100% asthenospermia semen samples. Under this condition a normal spermatozoa will absorb fluid. Resulting in bulging of the plasma membrane curling of the tail. The hypo-osmotic solution contains sodium citrate, fructose and distilled water. The solution

was stored at room temperature till used. A 1.0 ml of hypo-osmotic solution was mixed with 0.5 ml of undiluted semen and incubated at 37 0C for 20 mins. A drop of diluted semen was placed on a clean sterilized dry glass slide and covered with a cover slip. A total of 200 spermatozoa were counted in fields at 40X under phase contrast microscope and percentage of spermatozoa positive to HOS test (having coiled tails) was determined.

Eosin nigrosin staining (vitality staining)

Assessment techniques which are robust, reliable and as easy as possible to perform are fundamental to modern basic semen analysis. The assessment of sperm vitality is one of the basic elements of semen analysis, and is especially important in samples where many sperm are immotile, to distinguish between immotile dead sperm and immotile live sperm. The concept of using eosin to mark dead cells, which take up eosin, and nigrosin as a background stain, to increase the contrast between faintly stained cells and an otherwise bright background, is well known and widely used (*Blom, 1950; Williams and Pollak, 1950; Campbell et al., 1956; Beatty, 1957; Eliasson and Treichl, 1971; Dott and Foster, 1972; Dougherty et al., 1975; Eliasson, 1977; Mortimer, 1985; Mortimer et al., 1990;* World Health Organization, 1992; 1999). The simplified one-step technique, exposing sperm to a mixture of eosin and nigrosin, was introduced on boar, bull and ram sperm (*Campbell et al., 1956*), on rabbit sperm (*Beatty,1957)* and further evaluated for various mammalian sperm (*Dott and Foster, 1972)*. The technique was used on washed human sperm by Mortimer (*Mortimer et al., 1990*).

It is used to differentiate live and dead sperm in semen sample Then the sperms take eosin stain and appear as red whereas live sperms do not take the stain .This is taken as criteria to differentiate live and dead sperm cell .This vitality test should be carried out in semen samples with less than 50% motility **.**The eosin-nigrosin staining was performed by mixing 25 micro liters of semen with equal volume (25 micro litres) of the stain on above the mixture make a thin smears using another clean edged glass slide . Dry the slide in air and examine the smears under oil immersion objective (100 x magnifications). A total of 100 spermatozoa were then counted within 2 min after the addition of the stain. The results were expressed as the percentage of unstained (live) sperm and dead sperm take eosin nigrosin stain. Find out the percentage of live and dead sperms (vitality). A large number of live but immotile sperm cells may indicate an abnormality in the axoneme. And is considered as normal when percentage of live spermatozoa is more than 58%.

Leucocytes staining (white blood staining)

Elevated concentration of leucocytes in semen have been associated with genital tract infection, poor semen quality and IVF embryo transfer failure (*cohen et al.,1985).* According most human ejaculates contain leucocytes (*Wolff and Anderson, 1988; Aitken and West, 1990; Barratt et al, 1990),* the predominant cell type being the neutrophil. Excessive presence of these cells (leucocytospermia) may indicate the existence of reproductive tract infection. Furthermore, leucocytospermia may be associated with defects in the semen profile, including reductions in the volume of the ejaculate, sperm concentration, and sperm motility, as well as loss of sperm function as a result of oxidative stress (*Aitken et al, 1989; Aitken and West, 1990*) and/or secretion of cytotoxic cytokines (*Hill etal., 1987*). It is difficult to define a threshold concentration of leucocytes beyond which

fertility will be impaired. The impact of these cells depends upon the site at which the leucocytes enter the semen, the type leucocyte involved, and their state of activation. As a general rule, a normal ejaculate should not contain more than 5x 106 round cells/ml,while the number of leucocytes should not exceed 1x106/ml (WHO, 1992). When the semen contains more than 1x 106/ml white blood cells, microbiological tests should be performed to investigate if there is an accessory gland infection. Note: The absence of leucocytes does not exclude the possibility of an accessory gland infection.

WBC is used to estimate the leucocytes in semen. Presence of leucocytes (>1 million / ml) in the semen indicates the existence of an infection in male reproductive tract. WBC performed by mixing 20 µl of semen with equal volume (20µlitres) of stain incubated at room temperature (20-28ºc) for 5 mins. Then stain on a microscope slide and covered with coverslip. Count the peroxides cells (neutrophils) deep brown stain and rest of the round cells are unstained, which are of spermatogenic origin. Under a 40x magnification.

Macroscopic examination

A normal semen sample liquefies within 60 minutes at room temperature, although usually this occurs within 15 minutes. In some cases, complete liquefaction does not occur within 60 minutes, and this should be recorded. Normal semen samples may contain jelly-like grains (gelatinous bodies) which do not liquefy and do not appear to have any clinical significance.

The presence of mucous streaks may interfere with semen analysis. The sample must be well mixed in the original container and must not be shaken vigorously. During liquefaction, continuous gentle mixing or rotation of the sample container may reduce errors in determining sperm concentration (*de Ziegler et al., 1987*). If sperm motility is to be assessed at 37°C, the sample should be equilibrated to this temperature during liquefaction and mixing.

Liquefaction usually occurs within 10-20 minutes of collection. On a scale of 0-4 (4 being the normal value for a well liquefied sample), the failure to liquefy is usually a sign that there is inadequate secretion by the prostate of the proteolytic enzymes fibrinolysin, fibrinoginase and amino peptidase (10). Importantly, liquefaction should be differentiated from viscosity, as abnormalities in viscosity can be the result of abnormal prostate function and/or the use of an unsuitable type of plastic container.

High Viscosity can usually be reduced by repeated aspiration through an 18 or 19 gauge needle before or after diluting the semen with a suitable buffer or culture medium to reduce the amount of shear during physical disruption of the seminal gel.

Volume is measured with 2 to 5mL serologic pipettes; normal volume being between 2-6 mL.

Color is normally opaque or opalescent with translucent semen usually being oligospermic. The presence of a yellow hue or a very milky turbidity is associated with pyospermia while a rust colored or reddish semen is indicative of hematospermia.

pH a drop of semen is spread evenly onto the pH paper (range: pH 6.5-9.0). Compare colour change of the pH paper with the calibration strip while there is no further colour change and it is still moist.

Microscopic examination

Sperm concentration (normal > 20 million/mL) is usually measured either with a Makler chamber *(Rosenberg and Paulsen 1997)* a Neubauer hemocytometer .Use of the haemocytometer requires dilution of the semen sample, due to chamber depth which can be done with accuracy using volumetric pipettes with the diluent containing a fixative. No matter which method is used, several measurements on a well mixed specimen should be done and the mean of the results recorded. Adequate sampling is particularly important in severely oligozoospermic semen. When fewer than 10 million sperm/mL are present, we concentrate the specimen and re-suspend in a constant volume to increase the sensitivity of our measurements. Total sperm count (normal > 50 million) is then calculated by multiplying concentration times volume.

Motility (normal > 50%) is expressed as the percent of spermatozoa that have motion and the forward progression (normal > 3) is noted. A forward progression of 4 denotes spermatozoa rapidly moving in a straight line with no yaw or lateral movement; unfortunately, the *rapidity* (or linear velocity) of forward progression has not yet been standardized. A forward progression of 3 denotes spermatozoa similarly moving linearly but at a slower velocity. Sperm movement with a forward progression of 2 often exhibits angular displacement or yaw to varying degrees while a progression of 1 denotes only tail motion without progression.

Zero progression being no movement at all. The measurement of the duration of motility in seminal fluid is controversial. MacLeod (14) has pointed out that the seminal plasma is only a temporary transport medium and that evaluation of the duration of sperm motility *in vitro* in semen, is probably not physiologically significant as sperm rapidly lose their motility in the specimen container but remain motile for days in the mid cycle cervical mucus. If motility is less than 50% a viability stain is done using Eosin Y with Nigrosin as a counterstain. Greater then 50% of the sperm should be viable (i.e., non-stained). This is seen by bright field microscopy as red dye (Eosin Y) taken up by the head of most non-motile sperm.

Agglutination is also noted if present. Though clumping of spermatozoa can mean sperm antibodies are present, clumping usually only occurs around debris in the sample. However, if motile dimers, of either head to head or tail to tail associations are seen, this is diagnostic for the presence of antisperm antibodies in semen bound to the sperm. The quantification of red blood cells, if present, should also be made.

Morphology of sperm cell

Sperm have a head, a mid-piece and a tail, each component of which has particular morphologic characteristics. A comparison between the strict morphologic criteria of *Kruger et al., (1994)* and that of the WHO. The head of the normal human spermatozoa is ovoid in the frontal view and pyriform in the lateral view. Fixed and stained (Papanicolaou) sperm heads measure approximately 3-5 microns in length and 2-3 microns in width. The acrosome should make up somewhere between 40-70% of the normal sperm head. The mid-piece has a mitochondrial sheath and often excess cytoplasmic material from the developing spermatid. The tail principal piece is approximately 50-55 micron in length and varies in thickness from about 1 micron near the base to 0.1 microns at the tip of the end-piece as shown by electron microscopy.

The tail is composed of an axial core consisting of two central singlet microtubules surrounded by nine pairs of doublet microtubules, an outer ring of nine dense fibers surrounded by the fibrous sheath which define the principal piece of the tail. The various structural alterations that can occur in each region of the spermatozoa are shown in. Menkveld et al. have described an association between the "strict" morphologic evaluation of spermatozoa and the results of *in vitro* fertilization. Of particular note is the direct relationship between acrosome size and the frequency of both pregnancy and fertilization.

Structure of normal sperm cell

The shape of the HEAD is oval with regular outline and length is 4-5.5 microns, width is 2.5-3.5 microns. MIDPIECE is less than one third the width of head and length is 6-10 microns. TAIL and its shape looking like slender uncoiled, and with regular outline and length is 45 microns and other criteria is slightly amorphous (borderline) forms abnormal. No severe neck mid-piece or tail defects are allowed.

Structure of abnormal sperm cell

Head abnormalities

Large Oval Head: Spermatozoa is this category have three times larger head than normal spermatozoa with regular outline. The mid-piece and tail appear normal.

Small Head: Spermatozoa show a very tiny round head with irregular outline. The mid-piece and tail appear normal.

Tapering Head: The head length remains same but width decreases. Head assumes a "Cigar" shape. Mid-piece and tail is normal.

Pyriform Head: Spermatozoa under this category have heads in the shape of tear-drop. Tapered end coming to a point just above mid-piece. The mid-piece and tail appear normal.

Amorphous Head: The head of the spermatozoa show such bizzare shape that it cannot be classified in to any other category of head abnormality.

Double Head: Spermatozoa showing two distinct normal heads. It may be of different shapes also. The mid-piece and tail appear thick.

Round Head: The head of the spermatozoa are absolutely round possessing no acrosome and with regular outline.

Loose Heads: Spermatozoa seen as "free" or "loose" heads with little cytoplasm attached.

Mid piece abnormalites

Thinned out mid piece: Spermatozoa showing a normal head with mid piece that is thinned out (absent mitochondrial sheath) and continued with normal tail.

Bent Neck: The spermatozoa shows a clear bent at right angles to the longitudinal axis. The head and tail appear normal.

Cytoplasmic droplet: The cytoplasmic droplet is seen attached to the mid piece. The tail appears normal.

Tail abnormalities

Double tail: Spermatozoa show head with double tail. The mid piece appears to be double also.

Coiled tail: The is rounded over itself forming a compact coil. Coiling may be seen

only at the ends forming loose loop or compact.

Stumpy tail: Spermatozoa show a normal head with a short thick stumpy tail.

Headless: Spermatozoa actively motile showing only mid-piece and tail. A separate count of these headless spermatozoa should be done and expressed as percentage of headless spermatozoa to spermatozoa with head.

Empty tubules: These are very thin thread like tail elements of spermatozoa. Increased incidence indicates damage to spermatogenesis. The true significance of this is not yet know.

Result and Discussion

Forty semen sample were analyzed from the group of male subfertility who attended the NOVA IVF Fertility center for the primary fertility treatment. HOS (Hypo Osmotic Swelling Test) is a specific functional test Parameters, such as sperm using vitality test, wbc test and morphology and other microscopic and macroscopic analysis were done.

These forty specific sperm functional test analysis comparison were excluded from clinical comparison of IVF/ICSI, IUI treatment. These, 40 patient's analysis were divided into 4 groups and according to this sperm functional test parameters, Age group and also it has been graded for 4 individual as per the reference range.

A comparative analysis was done between HOS Test, Vitality, WBC and Morphology as well as different age groups with grading. Comparative analysis and percentage of results are shown in Table 1. The results are graded to reference range in Grade 1 normal, Grade 2 subnormal, Grade 3 Poor, Grade 4 Very Poor shown in Table 2.

Among the Forty male partners of different age (21 to >40) groups, the observed results were as follows, about 45 % functional test parameters is normal in the age group of 31-35, 32.5% functional test parameter is Subnormal in the age group of 26-30, 12.5% functional test parameter is poor in the age group of 21-25 and 10% functional parameter is also poor in the age group of 36-40, and 5% functional test parameter is very poor in the age group of >40%. Out of all >40 age group has very poor results in the sperm functional test.

This study demonstrates that subfertile men with reference range of HOS test values and specific functional test values such as vitality test, WBC test, morphology compared with different age groups values with grading have greater probability of causing pregnancy in the clinical treatment of IVF (In Vitro Fertilization)/ICSI (Intra Cytoplasmic Sperm Injection), IUI (Intrauterine Insemination). The HOS Test is easy to perform and not expensive, in contrast sperm count and motility fluctuate, the HOS test is stable over time (Shanis et al, 1992). And this studies 40 male infertility patients were done in functional test parameters have grading in different age groups 20 to >40. The percentage of HOS-reacted sperm (curled and swollen tails) and non reacted sperm were calculated by examining 100 spermatozoa. At least 60% swollen spermatozoa were considered normal.

And this functional test parameters done normal in the age group of 31-35, subnormal in the age group of 25-30, Poor in the age group of 21-25 and 36-40, Very poor in the age group of >40.As age increases, there is a significantly larger proportion of males with

very Poor HOS test. Under this result shows the impact structure of success fertility can reduce under the age factor of sperm quality. And this normal value of sperm quality in treatment for ICSI, Subnormal value of sperm quality for IUI, Poor and Very poor value of sperm quality in treatment of IUI, IVF/ICSI. As well as if its Poor or very poor specimen then this diagnosis will help as to treatment choice for constant couple, Weather it could be IUI or ICSI/IVF. Thus hypoosmotic swelling test if 60% swelling is a normal range and 50 to 60% swelling between normal and abnormal, less than 50% swelling definitely abnormal, that is, subfertile or infertile.

Initially, a study in which couples having a male partner with HOS Test scores <50% would be randomized to conventional IVF. IVF is an expensive and potentially risk procedure and conventional IVF without ICSI is an ineffective therapy (Check et al., 1995, Katsoff and Check, 1997). Only viable sperm cells, with chemically and physically intact membrane, undergo tail swelling due to water influx under Hypo Osmotic condition (Casper et al., 1996) a higher fertilization rate selected by the HOST

And the studies of HOS test is able to predict pregnancy rate and outcome in couples undergoing in vitro fertilization / intra cytoplasmic sperm injection and intrauterine insemination procedures (check et al.,1998, check et al.,2001, Tartagni et al., 2002). Our results are in agreement with those of other authors reporting a threshold value of 60% overall sperm swelling rate as an indicator of normal fertility potential of human spermatozoa (Shanis et al, 1992). These HOS Test is a promising method of identifying the live and dead spermatozoa for IVF/ICSI and IUI. The HOS Test is not included in standard semen analysis and is considered with the workup of unexplained infertility.

So, we may concluded that these Hypo Osmotic Swelling Test (HOS Test) and Combo such as vitality staining, WBC staining, Morphology analysis of semen is necessary to sub-fertile male who is taking infertility treatment as a primary clinical diagnosis .

The HOS test can be considered an easy, inexpensive, and reliable test for predicting male fertility potential and for identifying among subfertile men those who have a greater possibility of conceiving capacity of spermatozoa with timed intercourse following functional test parameter without Wasting time and money.

When the condition of severe Asthenozoospermia and complete absence of motility, HOS is the only clinical test and also way to find metabolically live spermatozoa for further assisted reproductive treatments.

The hypo-osmotic swelling (HOS) test evaluates the functional integrity of the sperm's plasma membrane and also serves as a useful indicator of fertility potential of sperm. The HOS test predicts membrane integrity by determining the ability of the sperm membrane to maintain equilibrium between the sperm cell and its environment.

Table.1 Reference range of functional test parameters

	NORMAL	SUBNORMAL	POOR QUALITY	VERY POOR
HOS TEST	>60	40-60	20 to 40	<20
VITALITY TEST	>58%	25-58%	15 to 25%	<15%
WBC	< 1 million	Is Genital	Tract	infection
MORPHOLOGY	4%	<4%	3%	

Table.2 Different age groups with gradient

AGE	G1	G2	G3	G4
21-25	3	1	0	1
26-30	4	5	2	2
31-35	8	6	1	1
36-40	2	1	1	0
>40	1	1	0	0

Figure.1 Structure of normal sperm

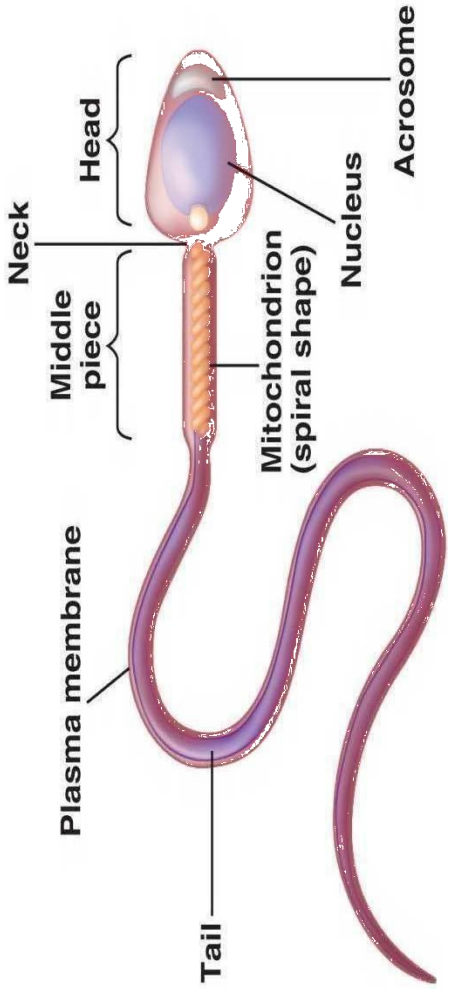


Figure.2 Normal Sperm and Abnormal sperms

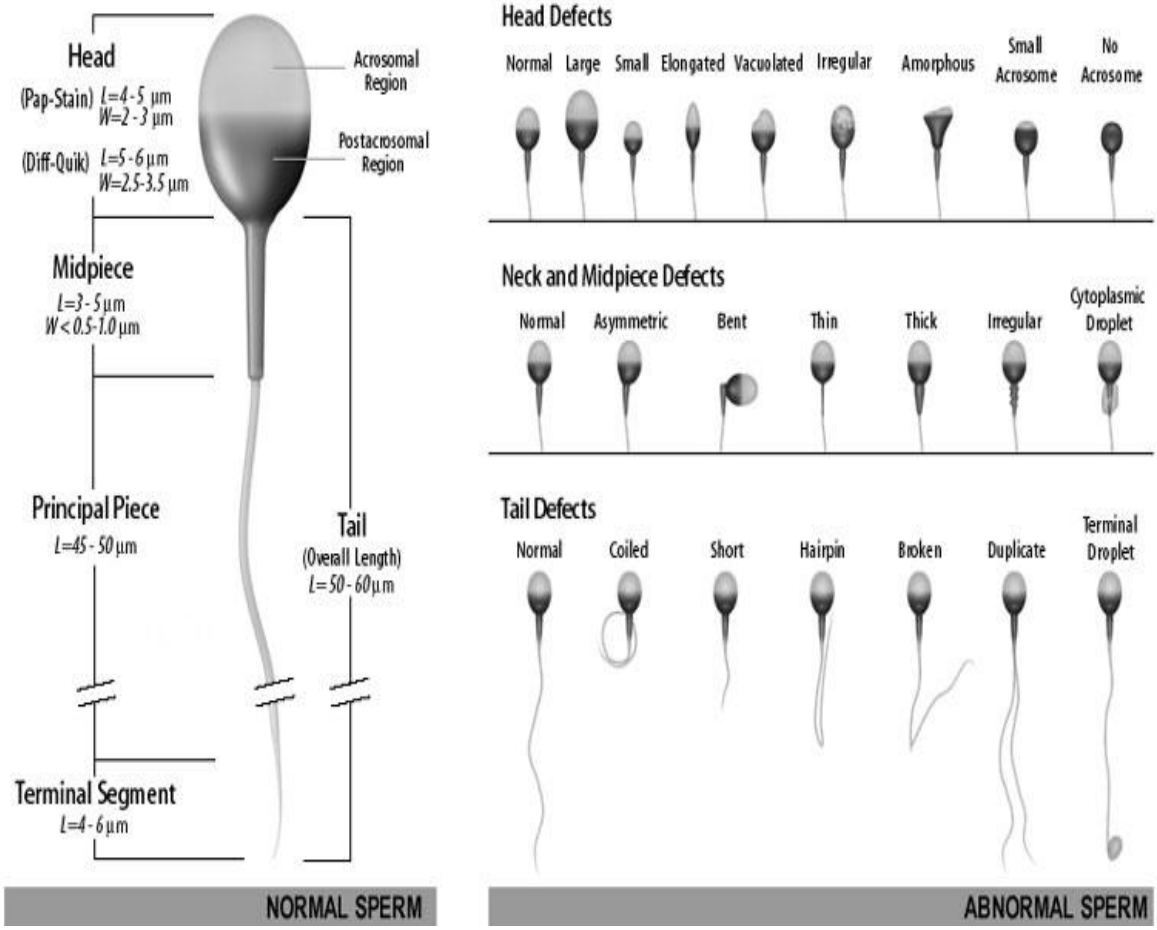


Figure.3 Experiment 1: Hypo Osmotic Swelling test (HOS Test)
Sperm (a) before Test (b) after HOS Test

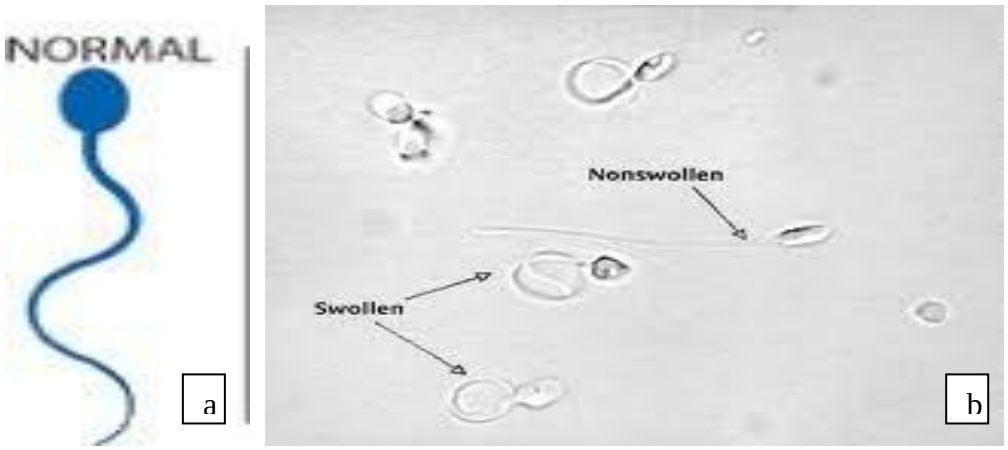


Figure.4 Sperm (a) before staining (b) after Eosin-nigrosin staining

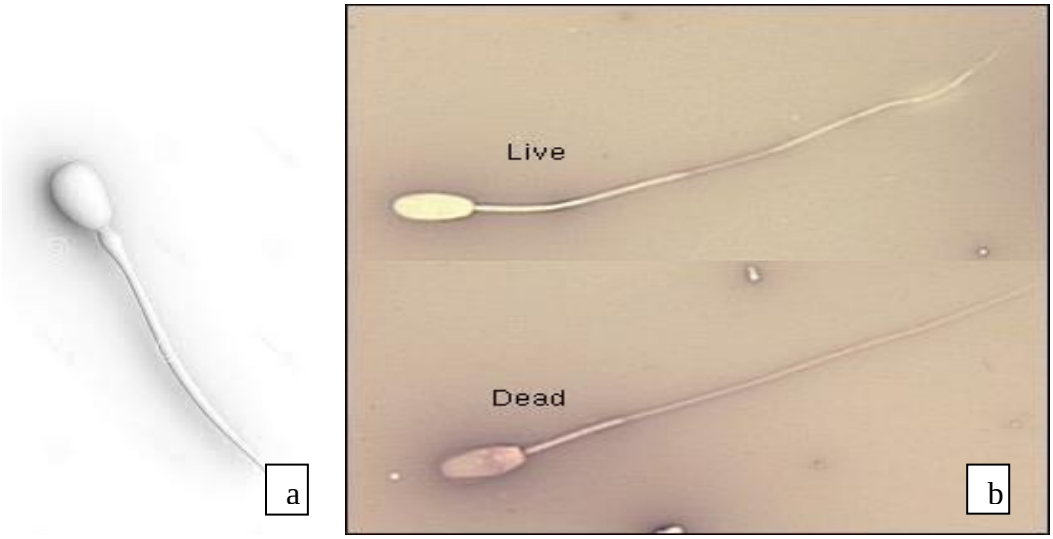
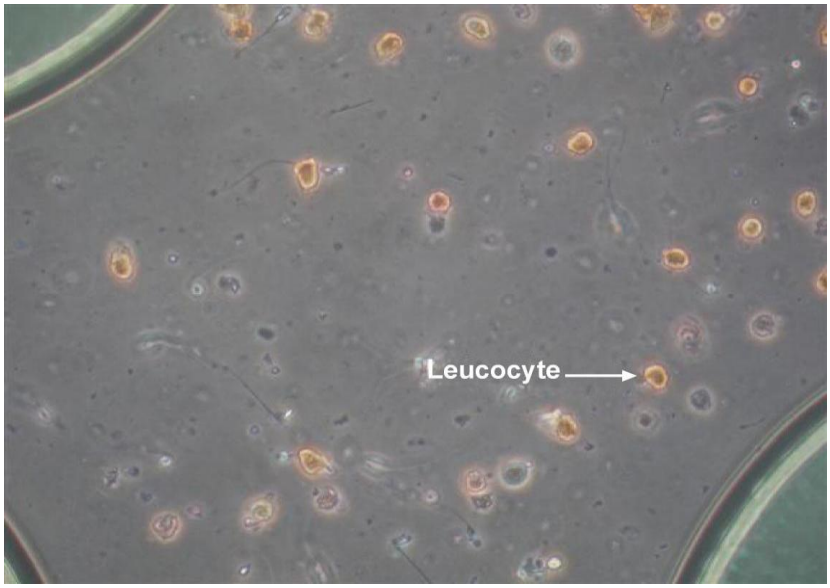


Figure.5 WBC Staining – Leucocytes (WBC test)



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