

# Fertility FOCUS

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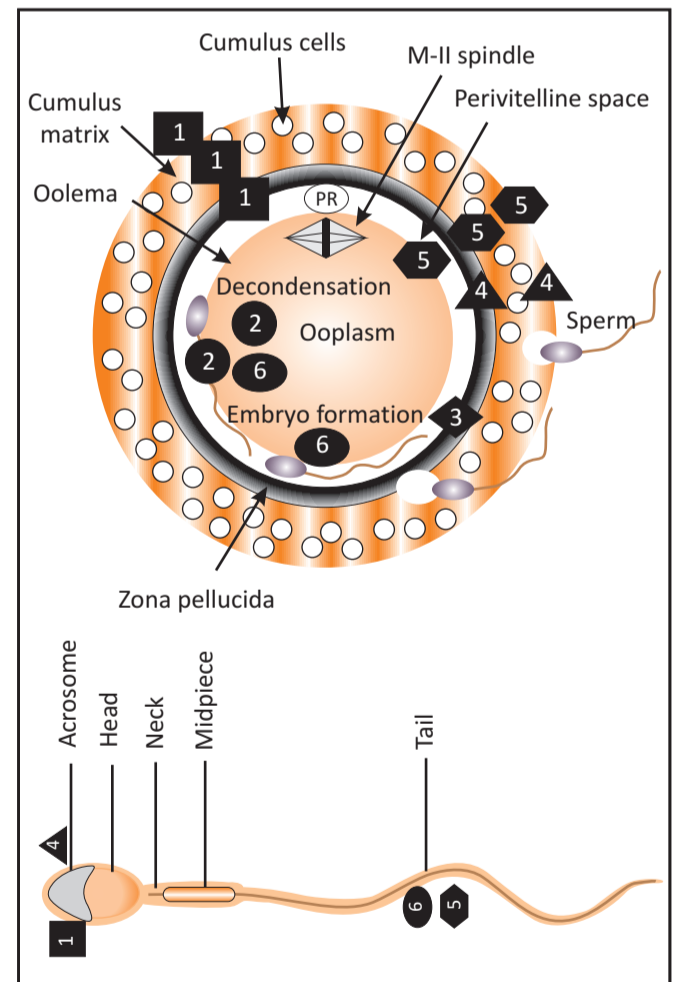
Routine semen analysis used for more than 50 years might not deliver better clinical information on fertility potential...

## Newer Diagnostic Sperm Tests In The Era Of ICSI

The complexity of involuntary childlessness can be understood in the context of female and male factors and a varied combination of both. In 20% of cases, male-related factors are responsible while in 30%, both the partners contribute to infertility<sup>1</sup>. Traditionally, analysis of sperm concentration, motility and morphology are performed to determine the etiology of male infertility or subfertility. However, routine use of light microscopy of 100-200 spermatozoa is associated a high margin of intralaboratory and interlaboratory variations<sup>2,3</sup>. In a meta-analysis of semen quality from 9612 presumably fertile men, 98 million/mL was considered as a normal sperm concentration<sup>1</sup> while sperm motility, assumed to be the “best” indicator of fertility, ranged from 53% to 62%<sup>4</sup>. Furthermore, semen analysis is widely influenced by an array of seasonal and geographic variations, which makes its reliability and quality control profoundly challenging.



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**Fig 1. Schematic illustration of the steps of the egg fertilization process that the older sperm function tests assess.** 1. Sperm morphology correlates with stages of egg cumulus and egg binding, and egg penetration and fertilization at IVF. 2. Sperm penetration assay assesses the ability of sperm to bind and penetrate the egg and decondense within it. 3. Hemizona assay examines sperm binding to the zona pellucida. 4. Acrosome reaction and the progesterone test assess the ability of sperm to penetrate cumulus and bind and penetrate the zona pellucida. 5. Hypo-osmotic swelling correlates with cumulus and egg binding and egg penetration and fertilization at IVF. 6. Reactive oxygen species evaluation correlates with sperm membrane, motility, and DNA integrity.

Natali A, Turek PJ et al Urology. 2011.

**Table 1. Lower references limits (5th centile) for semen characteristics (WHO 2010)**

Volume (mL)	1.5
Concentration (10 <sup>6</sup> sperm/mL)	15
Total sperm number (10 <sup>6</sup> /ejaculate)	39
Motility (% motile)	40
Forward progression	32
Morphology (% normal)	4
Viability/Vitality (% alive)	58%
White blood cells (10 <sup>6</sup> sperm/mL)	<1.0

Natali A, Turek PJ et al Urology. 2011.

WHO parameters of semen characteristics address only few aspects of sperm quality and function. During the last decades, several sperm function tests have shown promising potential. These include vital staining, biochemical analysis of semen, hypo-osmotic swelling test, sperm penetration assay, hemizona assay, antisperm antibody test, reactive oxygen species tests and computer-assisted sperm analysis. However, many of these tests are not routinely used.

**Delaying ICSI Up To 12 Hours Does Not Harm Outcome In Refrigerated Oocytes**

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## Newer Adjunctive Sperm Tests

### Sperm DNA Fragmentation

Recent research has linked biological correlate of fertility to the integrity of DNA within sperm chromosomes<sup>5</sup>. DNA damage or presence of altered sperm chromatin structure is attributed to four sources: (1) recombination deficiencies during the process of spermatogenesis, (2) dysfunctional protamination leading to abnormal spermatid maturation, (3) abortive apoptosis, and (4) oxidative stress<sup>5</sup>. Several assays examining sperm DNA integrity can be divided into (a) assays determining sperm chromatin structure, (b) assays for sperm DNA fragmentation, and (c) tests assessing sperm nuclear matrix<sup>5</sup>.

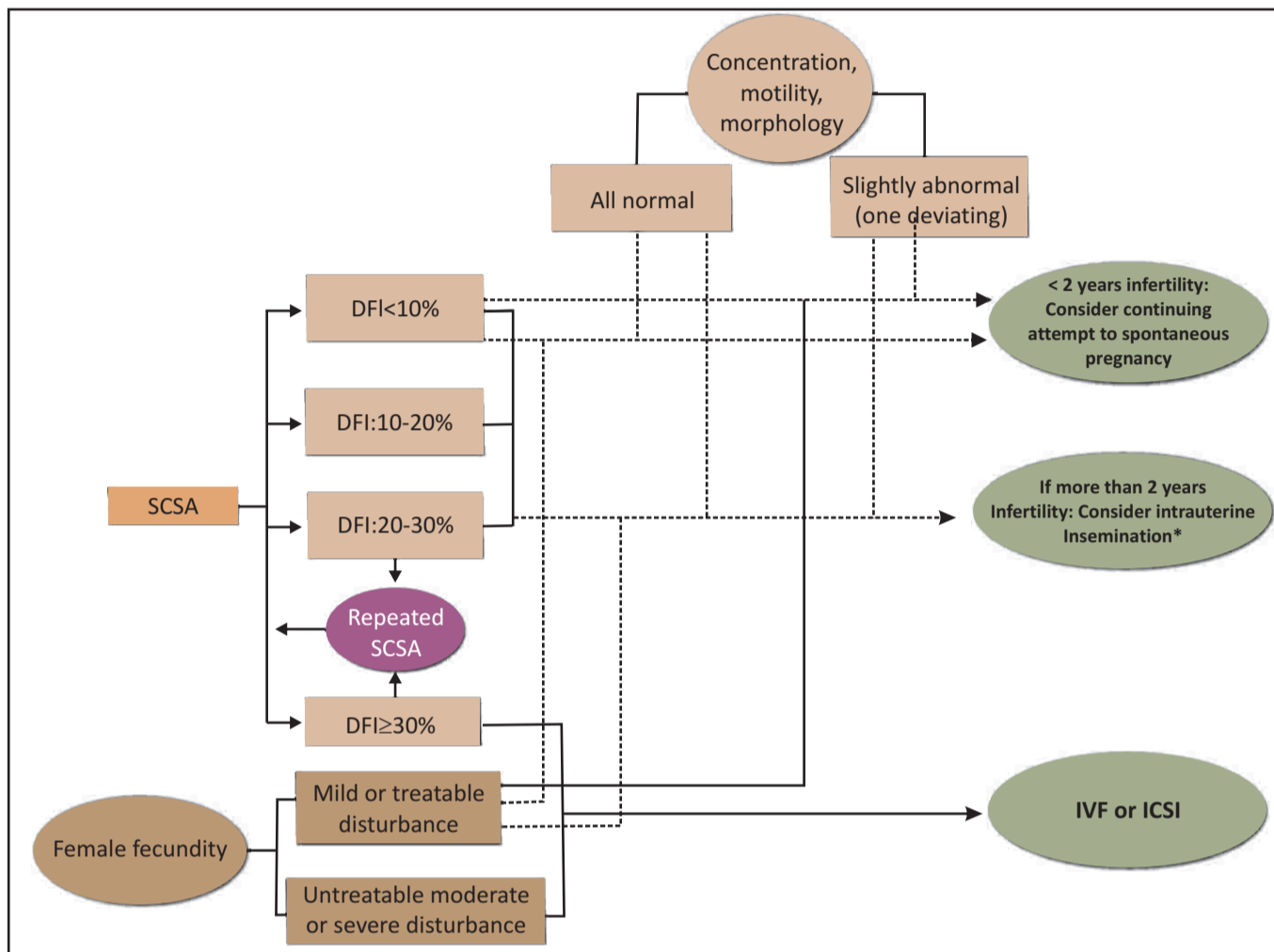


Fig 2. Flow chart for a possible use of sperm chromatin structure assay in diagnosis and treatment of infertility. SCSA, sperm chromatin structure assay. \*if  $DFI \leq 25\%$ , DFI, DNA fragmentation index.

Bungum M et al Asian Journal of Andrology 2011;13:69–75.

### Determining Sperm Chromatin Structure

Chromatin structural probes utilize highly sensitive nuclear dyes to examine DNA integrity. However, their cytochemical performance is fairly complex since several factors influence the process of DNA staining of chromatin by nuclear dyes. These are: (a) the secondary structure of DNA, (b) the regularity and density of chromatin packaging, and (c) the binding of DNA to chromatin proteins. Assays performed in this category are:

**ACRIDINE ORANGE:** This tool is inexpensive, and simple to perform. It measures *in situ* DNA susceptibility to acid-induced conformational helix-coil transition<sup>6</sup> (Fig. 3).

**ANILINE BLUE:** This tool stains proteins in loosely condensed chromatin.

**CHROMOMYCIN A:** This tool competes with protamines for association with DNA and staining relates to the degree of protamination in mature sperm. It is simple to perform<sup>7</sup>.

**TOLUIDINE BLUE:** This tool stains phosphate residues of loosely packed and fragmented sperm nuclear DNA<sup>8</sup>.

**SPERM CHROMATIN STRUCTURAL ASSAY (SCSA):** This tool measures *in situ* DNA susceptibility to acid induced conformational helix-coil changes with acridine orange fluorescence using automated cell sorting<sup>20</sup> (Fig. 3). SCSA is the most widely used assay, because of proven associations with clinical outcomes after natural conception and assisted reproductive. Although the procedure is expensive to perform, it offers small intra- and interassay variation.

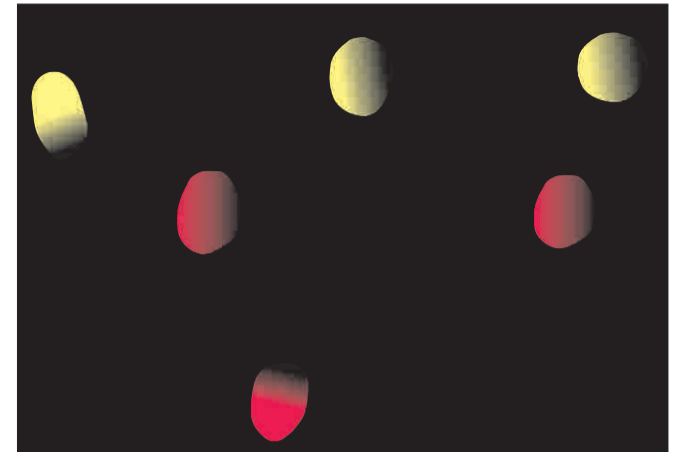


Fig 3. A common method of assessing sperm DNA fragmentation is to observe acridine orange (AO)-stained sperm that is exposed to 488-nm laser light. AO intercalated into double-stranded DNA fluoresces green and AO bound to single-stranded DNA fluoresces red.

### Tests Of Sperm DNA Fragmentation

Most frequent test to detect sperm DNA fragmentation include the Single-cell gel electrophoresis (Comet assay), the Terminal deoxynucleotidyl transferase-mediated dUDP nick end-labeling (TUNEL) assay, and the SCSA. All three label single or double-stranded DNA breaks.

#### *In situ* Nick translation

This is a quantification assay that measures incorporation of biotinylated deoxyuridine triphosphate (dUTP) at single-stranded DNA breaks (SSBs) within sperm DNA. This test is not widely used<sup>7</sup>.

#### Terminal deoxynucleotidyl transferase mediated dUTP Nick end labeling (TUNEL)

This assay can accurately detects double-stranded breaks (DSBs) in DNA through the incorporation of dUTP at DNA breaks (catalyzed by terminal deoxynucleotidyl transferase). Although *TUNEL* can efficiently detect DNA fragmentation in large population of sperms, the test may underestimate actual DNA fragmentation rates<sup>9</sup>. Also, the test is relatively labor-intensive and shows no clear clinical pregnancy outcome data<sup>10</sup>.

#### Single cell gel electrophoresis assay (COMET)

Single cell gel electrophoresis assay is a new, simple and sensitive method to evaluate DNA damage and repair at individual cell level. This assay can be performed on extremely small number of cells and results can be obtained within a relatively short time. This assay quantifies DNA SSBs and DSBs in single sperm after electrophoresis of fluorochrome-stained DNA<sup>11</sup>. Although, COMET is highly sensitive test, it can be difficult to standardize the comet tail length. In addition, assay correlation to clinical infertility outcomes is less apparent than with SCSA or TUNEL methods<sup>12</sup>. COMET is labor intensive.

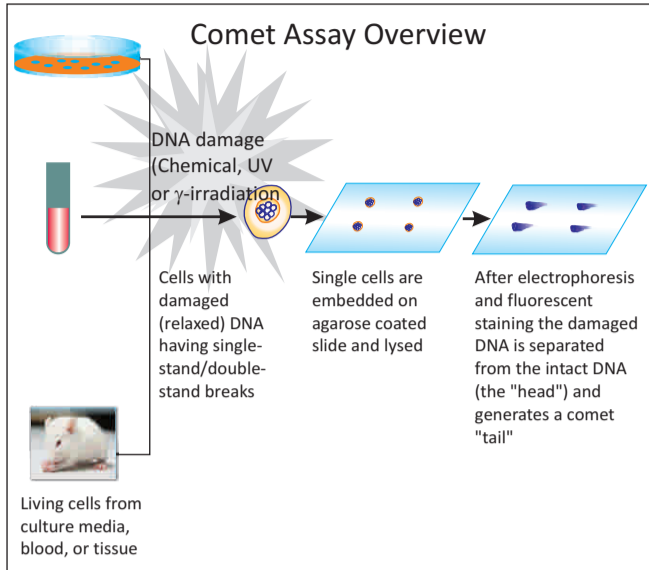


Fig 4. Individual cells are embedded in a thin agarose gel on a microscope slide. All cellular proteins are then removed from the cells by lysing. The DNA is allowed to unwind under alkaline/neutral conditions. Following the unwinding, the DNA undergoes electrophoresis, allowing the broken DNA fragments or damaged DNA to migrate away from the nucleus. After staining with a DNA-specific fluorescent dye such as ethidium bromide or propidium iodide, the gel is read for amount of fluorescence in head and tail and length of tail. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage.

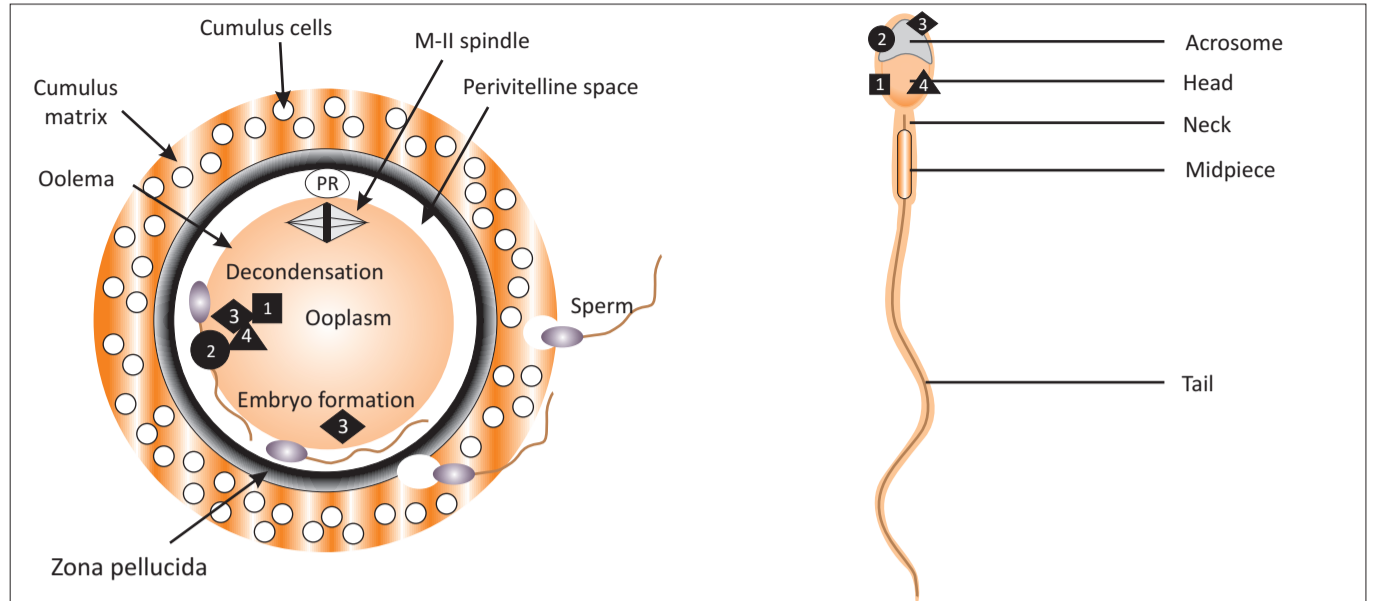


Fig 5. Schematic illustration of the steps of the egg fertilization process that the newer sperm function tests assess. 1. Sperm DNA fragmentation correlates with sperm decondensation and form embryos. 2. Sperm HA binding examines the ability of sperm to bind to the zona pellucida. 3. Ultrafine morphology correlates with the ability of sperm to decondense within the egg and form embryos. 4. Chromatin decondensation assesses the ability to sperm to decondense within the oocyte.

## Sperm nuclear matrix assays

This assay may be used to determine DNA organization in a semen sample by determining the degree of loop intact DNA deprived of chromatin proteins make around sperm nucleus matrix. Defining sperm DNA organization is important clinically since normal DNA organization is necessary for normal cellular function<sup>13</sup>.

### Sperm nuclear matrix stability

This assay assesses high-level DNA organization within the sperm nuclear matrix. It can detect aberrations in the ability of matrix to organize DNA into loop-domains. Clinical data on the assay is limited since the procedure is still being tested in its developmental stages.

### Sperm chromatin dispersion

Uniqueness of this assay is in fragmented DNA that do not produce the characteristic "halo" when mixed with aqueous agarose after treatment to remove nuclear proteins. Further utility of this assay in male infertility is being investigated.

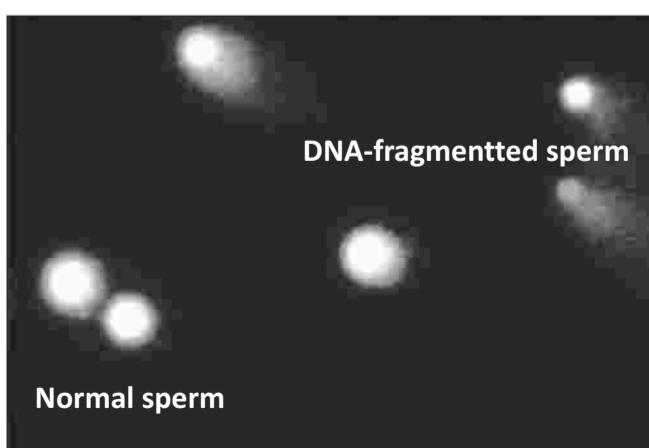


Table 2. Basics of common sperm DNA integrity assays

	Basis of Assay	Measured Parameter
<b>Direct assays</b>		
<b>TUNEL</b>	Adds labeled nucleotides to free DNA ends Template independent Labels SS and DS breaks	% Cells with labeled DNA
<b>COMET</b>	Electrophoresis of single sperm cells DNA fragments form tail Intact DNA stays in head Alkaline COMET Alkaline conditions, denatures all DNA Identifies both DS and SS breaks Neutral COMET Does not denature DNA Identifies DS breaks, maybe some SS breaks	% Sperm with long tails (tail length, % of DNA in tail)
<b>In situ nick translation</b>	Incorporates biotinylated dUTP at SS DNA Breaks with DNA polymerase I Template-dependent Labels SS breaks, not DS breaks	% Cells with incorporated dUTP (fluorescent cells).
<b>Indirect assays</b>		
<b>DNA break detection FISH</b>	Denatures nicked DNA Whole genome probes bind to SS DNA	Amount of fluorescence proportional to number of DNA breaks
<b>SCD</b>	Individual cells immersed in agarose Denatured with acid then lysed Normal sperm produce halo	% Sperm with small or absent halos
<b>Acridine orange flow cytometric assays</b>	Mild acid treatment denatures DNA with SS or DS breaks Acridine orange binds to DNA DS DNA (nondenatured) fluoresces green SS DNA (denatured) fluoresces red Flow cytometry counts thousands of cells	DFI-the percentage of sperm with a ratio of red to (red + green) fluorescence greater than the main cell population
<b>Acridine orange test</b>	Same as above, hand-counting of green and red cells	% Cells with red fluorescence

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Table 3. Advantages and disadvantages of various DNA integrity assays

	Pros	Cons
<b>Direct assays</b>		
<b>TUNEL</b>	Can perform on few sperm Expensive equipment not required	Thresholds not standardized Variable assay protocols
<b>COMET</b>	Sensitive Can perform on few sperm Alkaline: identifies all breaks Neutral: may identify more clinically relevant breaks	Labor intensive Requires imaging software Variable assay protocols Alkaline: may identify clinically unimportant fragmentation May induce breaks at "alkaline-labile" sites Neutral: less sensitive
<b>In situ nick translation</b>	Simple	Unclear thresholds, Less sensitive
<b>Indirect assays</b>		
<b>DNA break detection FISH</b>	Can perform on few sperm	Limited clinical data
<b>SCD</b>	Easy, can use bright-field microscopy	Limited clinical data
<b>Acridine orange flow cytometric assays</b>	Many cells rapidly examined Most published studies reproducible	Expensive equipment required Small variations in lab conditions affect results Calculations involve qualitative decisions
<b>Manual acridine orange test</b>	Simple	Difficulty with indistinct colors, rapid fading, heterogeneous staining

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# Fertility Myths And Illusory Benefits Of Healthy Habits In Young People

**Research shows that people cannot recognize factors that have no effect on fertility. Most believe in myths and “healthy behavior” rituals and trust these to actually increase a woman’s fertility potential. Fertility awareness campaigns are needed to address such false beliefs**

**R**esearch has highlighted that knowledge is a key factor associated with fertility self-care. A global survey of almost 17,500 people (most of childbearing age) from 10 countries showed poor level of knowledge regarding fertility and biology of reproduction. Many people overestimate the chances of pregnancy at the time of ovulation, have little awareness of their own fertile period, and are not aware of the nuances of infertility.

It is vital to disseminate knowledge about the biological process of reproduction. For instance, information on when a woman is most fertile, how long sperm survives, when is the best time for unprotected intercourse and the difficulties associated with conception. However, equally important is for people to be aware of factors that could reduce the chances of conception, and how lack of knowledge in this area makes people unintentionally reduce their own future prospects of fertility.

**Erroneous belief is an important factor affecting fertility self-care. For instance, people falsely believe that they ‘increase’ their fertility by not smoking rather than simply avoiding decrements in fertility due to smoking.**

In a recent investigation, Bunting and Biovin assessed knowledge of fertility more broadly in young people. The authors investigated three areas of knowledge, namely risk factors associated with female infertility, beliefs in false fertility myths, and beliefs in the illusory benefits of healthy habits on female fertility. Study enrolled 149 subjects consisting of 110 female and 39 male postgraduate and undergraduate university students (average age 24.01, SD=7.81). Knowledge scores were based on a task that required participants to estimate the effect a factor would have on a group of 100 women trying to get pregnant. Items (n=21) were grouped according to three categories: risk factors, myths, and healthy habits (e.g. being normal weight; 7 items).

Results showed that young people were significantly better at correctly identifying the effects of risks compared with null effects of healthy habits (P<0.001) or fertility myths (P<0.001). In the study, most participants identified all high risk factors that decreased the chance of pregnancy. Age over 45 years was considered the highest risk while age within 35–39 years had the least impact. Most participants correlated myths and healthy habits with successful pregnancy rates. Consumption of five portions of fruits and

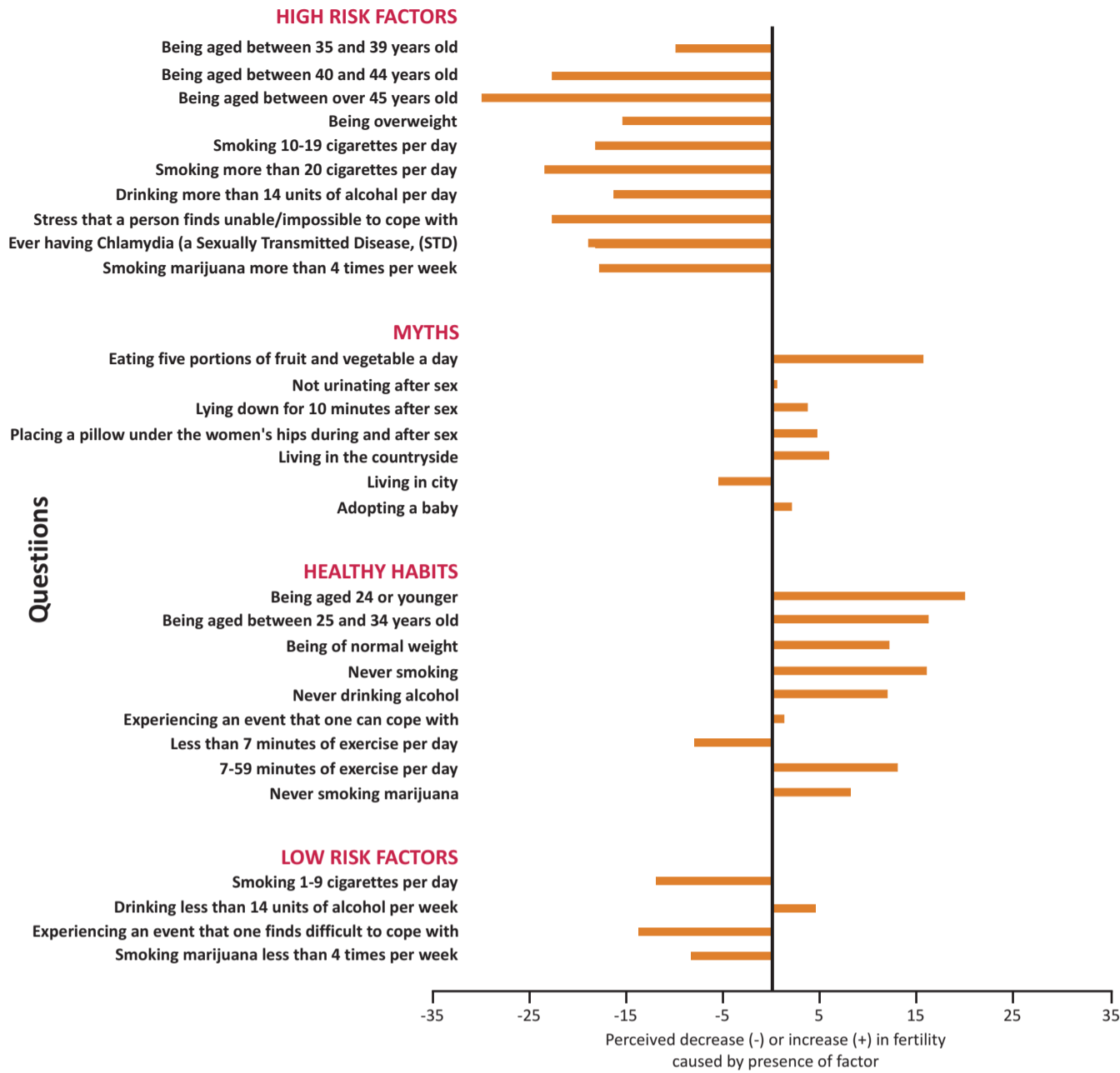


Fig 8. Pregnancy gain/loss scores per item, according to the category in a survey of knowledge about female infertility in young people.

vegetables had the largest score with positive pregnancy outcome while living in the city decreased the number of women getting pregnant.

At the same time, participants believed that living in countryside actually increased the likelihood of conception. Interestingly, other than exercising less than 7 minutes per day, all the healthy behaviors were rated as having a positive influence on the pregnancy rate. Again, being 24 years of age was associated with a positive gain score of 19.56 while the ability to cope with stressful events showed the smallest gain (1.24).

The study also showed that besides the myths, participants wrongly believed that one could be more fertile by 'not doing' something unhealthy (e.g. not consuming alcohol). This assumption is false since following healthy lifestyles help reduce the exposure to risk rather than promoting health. These findings suggest that when faced with a fertility problem, people often engage in ineffective behaviors that usually delay the process of seeking professional intervention.

It is not surprising that people who maintain a healthy lifestyle are often astonished that they should be infertile given that they were the healthiest in their family. Indeed, further research is required to establish the impact of incorrect information on personal risk perception and decision-making process especially when couples are faced with difficulties in conception.

**In the present study, woman's age was associated with the largest pregnancy loss score (29.43%). Participants identified that fertility declined from 35 years of age. However, in Western countries, there has been a steady increase in the number of women having children over 35 years of age. Indeed, studies on general versus personal risk perception show that in the process of decision-making, people do not apply risk to themselves. Having access to right information may only be the first step in the process of behavior change.**

## Effect Of Body Mass Index On In Vitro Fertilization Outcomes In Women

Obesity has become a major health problem across the world. In women, it is known to cause anovulation, subfecundity, increased risk of fetal anomalies and miscarriage rates. However, in women going for assisted reproduction the effects of obesity on egg quality, embryo quality, clinical pregnancy, live birth rates are controversial. In a recent analysis, Sathya and Balasubramanyam assessed the effect of women's body mass index (BMI) on the reproductive outcome of non donor IVF/ICSI. The effects of BMI on their gonadotrophin levels (day 2 LH, FSH), gonadotrophin dose required for ovarian stimulation, endometrial thickness and oocyte/embryo quality were examined, after correcting for age and poor ovarian reserve.

Retrospective medical records of 308 women undergoing non donor IVF cycles were examined. Subjects were classified into three groups: normal weight (BMI<25 kg/m<sup>2</sup>), overweight (BMI>25 <30 kg/m<sup>2</sup>) and obese (BMI>30 kg/m<sup>2</sup>). All women underwent controlled ovarian hyper stimulation using long agonist protocol. There were 88 (28.6%) in the normal weight group, 147 (47.7%) in the overweight and 73 (23.7%) in the obese group. All three groups were comparable with respect to age, duration of infertility, female and male causes of infertility. The three groups were similar with respect to day 2 LH/FSH levels, endometrial thickness and gonadotrophin requirements, oocyte quality, fertilization, cleavage rates, number of good quality embryos and clinical pregnancy rates. An increased body mass index in women was not associated with adverse IVF outcome. However, preconceptual counselling for obese women is necessary as weight reduction helps in reducing pregnancy-related complications.

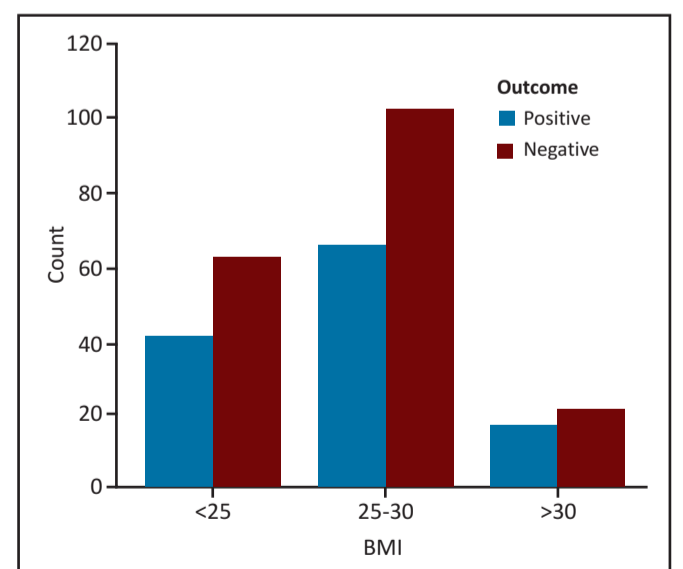


Fig 9. Effect of BMI on pregnancy outcome <25-34+, 38-, (total = 72), 25-30-52+, 64-, (total = 116), >30- 12+, 14-, (total = 26), P=0.95 (not significant). Correlation studies between the body mass indices and the pregnancy rates, implantation rates and fertilization rates failed to show any significant association.

J Hum Reprod Sci. 2010; 3(3): 135-138.

# Comprehensive Algorithm For The Evaluation, Diagnosis And Treatment Of The Infertile Man Presenting With Low Semen Volume

**Table 5. Etiology Of Low Semen Volume**

Etiology	Features
<b>Artifact</b>	Short abstinence period Incomplete collection
<b>Psychogenic</b>	Anorgasmia
<b>Pathologic</b>	Retrograde ejaculation Structural (damage to bladder neck) Functional (nerve and neurotransmitter) Failure of emission (nerve) Ejaculatory duct obstruction Congenital ( $\pm$ seminal vesicles or vas anomalies) Acquired Agenesis or aplasia of the seminal vesicles, prostate Seminal vesicle disease (infection, cysts, ADPKD) Hypogonadism

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**Table 6. The Patient's History**

**Is This An Artifact?**

Question the patient about the abstinence period, collection methods used (masturbation, SCD, etc) and completeness of collection. Asking whether the patient feels that the volume of ejaculate produced during specimen collection is similar to the volume produced during normal sexual activity may help to identify patients whose semen volume is low only during specimen collection.

**Is There A Psychogenic Cause?**

The history should carefully assess overall sexual function (libido, erection, orgasm, ejaculation) to identify potential contributing psychogenic conditions. One important factor to determine is if the patient actually has an orgasm.

**Is There A Pathologic Cause?**

The pathologic causes include retrograde ejaculation, failure of emission and alterations in the Wolffian duct structures (seminal vesicles, ejaculatory ducts and vas deferens).

**Is There Evidence For Retrograde Ejaculation Or Failure Of Emission?**

A history of cloudy urine following ejaculation is often associated with RE, and this should be elicited. History of surgery, trauma or disease that might affect the sympathetic nerves or the bladder neck should be elicited. A finding of absent ejaculate must prompt questioning regarding spinal or neurologic disease, and previous prostate surgery. Other symptoms of neurologic dysfunction (leg weakness, bladder/bowel dysfunction) should also be sought.

**Is There Evidence Of An EDO Or Absence Of The Wolffian Ducts?**

A history of prostatic surgery or infection, and symptoms such as pain with ejaculation or hematospermia, can be associated with EDO. While most patients with CBAVD do not have clinical CF, history of respiratory illness/symptoms, as well as a family history of CF and infertility, is useful.

**Are There Symptoms Of Hypogonadism?**

Low testosterone levels can be associated with various symptoms of hypogonadism, and these should be elicited (low energy, mood changes, weakness, ED, decreased libido).

SCD=silastic seminal collection device; RE=retrograde ejaculation; EDO= ejaculatory duct obstruction; CBAVD=Congenital unilateral absence of the vas deferens; CBAVD=congenital bilateral absence of the vas deferens; CF=cystic fibrosis; ED = erectile dysfunction.

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Ejaculate volume is an important component of the semen analysis and evaluation of the infertile man, but is often overlooked if other abnormalities are also present on semen analysis (e.g., low sperm count). A careful history and physical examination can help identify most causes, and can help guide subsequent investigations. Treatment to correct the problem may be possible in some patients and, in others, identification of important medical conditions may occur.

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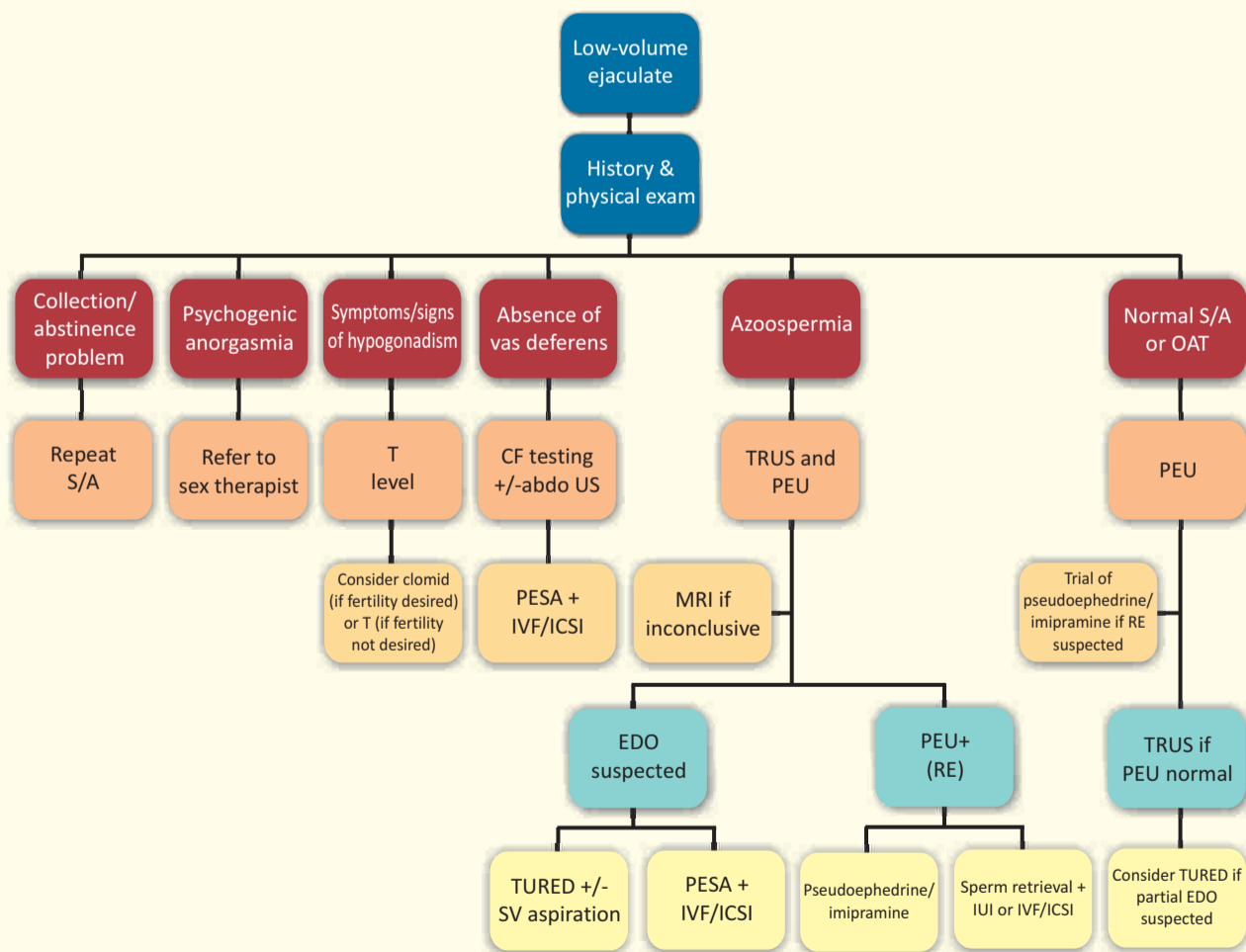


Fig 10. Evaluation And Treatment Of Low-volume Ejaculation S/A = semen analysis; OAT = oligoastheneratozoospermia; Abdo US = abdominal ultrasound; CF = cystic fibrosis; PEU = post-ejaculate urinalysis; TRUS = transrectal ultrasound; PESA = percutaneous epididymal sperm aspiration; ICSI = intracytoplasmic sperm injection; RE = retrograde ejaculation; TURED = transurethral resection of the ejaculatory ducts; SV = seminal vesicle; IUI = intrauterine insemination; EDO = ejaculatory duct obstruction.  
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