

Semen analysis

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Introduction

Semen analysis is an integral part of the workup of couples consulting for infertility. The availability of semen renders possible direct examination of male germ cells, giving precious data that are not accessible for female germ cells.

Semen analysis includes the examination of :

- Spermatozoa
- Other cells present in semen
- Seminal fluid

Altogether these data give indications on the testicular function and of the integrity of the male genital tract.

Type of assays

1. Descriptive assay:	spermogram
2. Functional assays:	penetration of cervical mucus (postcoital test, in vitro penetration assay) binding of spz to the zona pellucida fusion of spz with zona-free hamster oocyte hyposmotic swelling of the flagella
3. Immunological assay:	mixed agglutination test immunobead test sperm immobilization in cervical mucus

Spermogram

Definitions of semen classifications

Normozoospermia	When all the spermatozoal parameters are normal together with normal seminal plasma ,WBCs and there is no agglutination.
Oligozoospermia	When sperm concentration is < 20 million/ml.
Asthenozoospermia	Fewer than 50% spermatozoa with forward progression(categories (a) and (b) or fewer than 25% spermatozoa with category (a) movement.
Teratozoospermia	Fewer than 50% spermatozoa with normal morphology.

Oligoasthenoteratozoospermia	Signifies disturbance of all the three variables (combination of only two prefixes may also be used).
Azoospermia	No spermatozoa in the ejaculate.
Aspermia	No ejaculate.

Normal values of semen variables

Normal values of semen parameters have been issued by WHO in 1992 that are generally used as reference. Ideally each laboratory should set its own normal values reflecting the specific population analyzed, but this is practically limited by the availability of semen from proven fertile men who have recently achieved a pregnancy.

It should be emphasized that semen is an exception amongst biological fluids since its parameters display very wide intra and inter-individual variations. Therefore semen analysis should be repeated to take intra-individual variations over time into account and confirm abnormal parameters.

Normal values of semen variables^a

Standard tests	Normal values
Volume	2.0 ml or more
pH	7.2-7.8
Sperm concentration	20x10 ⁶ spermatozoa/ml or more
Total sperm count	40x10 ⁶ spermatozoa or more
Motility	50% or more with forward progression or 25% or more with rapid progression within 60 min after collection
Morphology	30% or more with normal morphology ^b
Vitality	75% or more live
White blood cells	Fewer than 1x10 ⁶ /ml
Immunological tests	
Immunobead test	Fewer than 20% spermatozoa with adherent particles
MAR test	Fewer than 10% spermatozoa with adherent particles
Seminal plasma biochemical analysis	
Epididymal markers	
a-glucosidase (neutral)	20 mU or more per ejaculate
Carnitine	0.8-2.9 mmole per ejaculate
Prostate markers	
Zinc (total)	2.4 mmole or more per ejaculate

Citric acid (total)	52 mmole or more per ejaculate
Acid phosphatase (total)	200 U or more per ejaculate
Seminal vesicle marker	
Fructose (total)	13 mmole or more per ejaculate

^aWHO manual, 3rd edition, 1992.

^bempirical reference value.

Scheme of spermogram

- Spermatozoa parameters: the spermatozoa parameters assessed in the spermogram are the number of sperm cells, the viability, the motility and the morphology of the sperm population.
- Immunological analysis: immunobead or mixed-agglutination (MAR) tests allow to detect anti-spermatozoa-antibodies
- Seminal fluid parameters: biochemical assays of markers for prostate, seminal vesicles and epididymis assess the function of these accessory glands.

Basic information

- Time of abstinence (3-5 days)
- Sampling (complete)
- Time of sampling

Macroscopic analysis

- pH, measured with pH paper
- volume, measured by weight
- appearance (abnormal smell, colour or viscosity)
- liquefaction time (above 60 minutes, treat with bromelin)
- seeding for bacterial analysis

Microscopic analysis on live sperm

Search for:

- Aggregated spermatozoa
- Epithelial cells
- White blood cells
- Bacteria

Sperm concentration, total number and viability

The major part of the ejaculate volume is contributed by secretions from the accessory glands (seminal vesicles and prostate), so the ejaculate volume is not directly related to spermatogenesis and hence the sperm cell concentration (sperm cells/ml) varies according to the ejaculate volume. The total number of spermatozoa per ejaculate reflects the spermatogenesis and is related to the time of sexual abstinence before collection. In normal situation spermatogenesis is considered to be a constant process over time and therefore the total number of sperm per ejaculate should increase with abstinence time. The ejaculate volume is related to the secretory function of the seminal vesicles and prostate.

Decreased ejaculate volume and increased sperm concentration reflects impaired accessory glands function. Vital staining of the spermatozoa allows to quantitate the fraction of living cells independently from their motility.

Spermatozoa motility

The fraction of motile sperm in semen is measured either by manual counting or using a computer assisted semen analysis (CASA) system. Motility is assessed at the time of semen liquefaction and after 1 and 3 hours to detect asthenozoospermia. Manual counting classifies sperm cells into 4 categories (immotile, locally motile, non linear and linear motile) using qualitative subjective criteria of selection. Many infertility centers now use CASA systems for objective measurements of sperm motion and positive correlations have been found between motion parameters such as the amplitude of lateral head displacement, curvilinear velocity, linearity and straight-line velocity and fertilization rates in vitro but the threshold levels for these motion characteristics have not yet been established to meet a general consensus.

Bacteriological analysis

Direct measurement of infectious contamination is obtained from bacteriological cultures of both aerobic and anaerobic germs. In normal conditions semen is not sterile but rather colonized at low levels by a variety of germs. Recent studies have shown that bacterial colonization did not have negative impact on sperm-cervical mucus interaction.

Biochemical analysis

Biochemical analysis of secretion components from prostate, seminal vesicles and epididymis in semen give informations about the functional state of these organs. These markers include fructose as seminal vesicles marker, zinc or acid phosphatase as prostate marker and carnitine as epididymis marker. Zinc can be measured by colorimetric assay while fructose and carnitine are measured using enzymatic assays.

Anti-sperm antibodies

The presence of anti-sperm antibodies in semen can alter the sperm fertilizing ability. Spermatogenesis starts at puberty, after the "education" of the immune system to recognize self antigens is finished and are therefore immunogenic. Under normal circumstances they are protected from the man's immune system by the hemato-testicular barrier that separates the inner part of seminiferous tubules from the blood. When this barrier is ruptured sperm cells induce the synthesis of anti-sperm antibodies. Antibodies adsorbed on the sperm surface can be detected by immunological assays using secondary, Ig class-directed antibodies that are coupled to beads. The percentage of sperm adhering to the beads reflects in a semi-quantitative manner the presence of anti-sperm antibodies.

We currently use Mar-test kits to detect anti sperm-IgG in semen. Positive and dubious samples are subsequently tested for anti-sperm IgA. IgA antibodies are more significant clinically, but very rarely occur without associated IgG. Therefore the test of anti-sperm IgG antibodies in semen is sufficient for the first screening procedure. Anti-sperm IgG can be tested in serum but this is of little benefit as serum anti-sperm IgG do not correlate with anti-sperm Ig in semen and do not influence fertility prognosis.

Morphology analysis

The evaluation of sperm morphology is performed after Papanicolaou or similar staining and consists in detailed examination of 100 sperm cells as well as other cells present in the ejaculate, including leucocytes and immature sperm cells. Sperm cells represent a unique population where over 50% of the cells can have morphological defects in normal fertile individuals. These defects affect the head, midpiece or the flagella of the sperm cell. The percentage of sperm with normal morphology will be recorded as well as the individual abnormalities, in order to detect predominant abnormalities, which suggest genetic defects affecting spermatogenesis. The rare cases of monomorphic teratozoospermia as well as severe asthenozoospermia can be subjected to EM analysis to detect specific defects at the ultrastructural level, particularly in the flagella where abnormal microtubule assembly can be found as in the immotile cilia syndrome.

In recent years, Menkveld, Kruger and colleagues have described strict criteria for spermatozoa morphology assessment with which they obtained good predictive value for in vitro fertilization. They report normal in vitro fertilization rates for cases with > 14% normal sperm morphology. The need to follow Kruger's criteria of selection of normal sperm morphology is still debated. Nevertheless, numerous studies have now clearly established that sperm morphology is an important parameter in the evaluation of sperm fertility.

The evaluation of sperm morphology also includes the identification of other cell types present in semen such as immature sperm cells and leukocytes. The presence of immature germ cells in semen indicates spermatogenesis dysfunction at the testicular level whereas leukocytes in concentrations exceeding $1 \times 10^3/\text{ml}$ indicate inflammatory conditions possibly related to infection.

Distinction between leucocytes and spermatogenesis cells

The distinction between spermatogenesis cells and leucocytes is not always obvious, and it is important particularly in cases of azoospermia. Indeed, in azoospermia, the presence of spermatogenesis cells indicates a testicular malfunction, while the presence of leucocytes in the absence of any spermatogenesis cells suggests that the azoospermia might be due to an obstruction problem.

Spermatogenesis cells present in semen are usually degenerating, and can sometimes be confused with leucocytes. In particular, multinucleated spermatids can be confused with polymorphonuclear leucocytes (PMN). Staining of endogenous peroxidases present in PMN can help to distinguish between these two cell types.

Sperm – cervical mucus interactions

Cervical mucus is the major physical barrier that sperm cells have to cross to access to the female upper genital tract. Less than 1% of the sperm deposited in the vagina successfully penetrate the cervical mucus. Evaluation of sperm-cervical mucus interactions include the post-coital test, the sperm cervical mucus contact test and the in vitro sperm-cervical mucus penetration assay.

Post-coital test

The post-coital test is the analysis of cervical mucus a few hours after intercourse. It reflects the physiologic situation in vivo and assess both the quality of cervical mucus and the penetration ability of sperm. The quality of cervical mucus varies during the cycle and is favourably influenced by estrogens, becoming more abundant and fluid at the time of ovulation. Therefore post-coital tests are scheduled just before ovulation as determined by basal body temperature, or more accurately by follicular sizing by ultrasonography. The number of motile sperm per high power microscopic field is recorded and the test is considered positive when 10 or more motile sperm are found per field according to WHO guidelines. Cervical mucus evaluation (including volume, consistency, ferning, spinnbarkeit, cellularity and pH) is of utmost importance for the interpretation of post-coital test results with respect to sperm function. A decreased number of sperm in cervical mucus when the cervical mucus score is low reflects inadequate mucus rather than impaired sperm function. Cervical mucus is colonized by sperm that are stored for several hours in cervical crypts. Sperm cells then gradually migrate through the cervix. Therefore sperm are present in cervical mucus constantly for at least 12h following intercourse and the timing of post-coital test (6-12h after intercourse) allows to test the viability of sperm in this environment. Abnormal penetration of cervical mucus by sperm has been associated with the presence of immobilizing anti-sperm antibodies, and repeatedly abnormal post-coital test with normal score cervical mucus associated with normal sperm concentration and motility should be investigated by additional tests such as anti-sperm antibodies detection in semen, sperm-cervical mucus contact test and in vitro cervical mucus penetration assay.

The post-coital test has been used for several decades but is reported to be inaccurate and to lack consensus normal values and methodology. Part of the problem may be due to the non-homogeneous nature of the cervical mucus that prevents quantitative determination of sperm concentration. The post-coital test however remains an inexpensive and

noninvasive procedure that gives information about the occurrence of ejaculation and the ability of the sperm cells to function within the cervical environment.

Sperm-cervical mucus contact test

Sperm-cervical mucus contact test consists in mixing semen and cervical mucus in vitro and measuring the appearance of immobilized "shaking" motile sperm. This is interpreted as the adherence of antibody-coated sperm cells to cervical mucus and was shown to correlate with semen anti-sperm antibodies and pregnancy rate. This test can be performed in parallel with donor semen or donor cervical mucus, and therefore allows to discriminate between a male versus female origin of the sperm immobilizing factor.

In vitro cervical mucus penetration test

The third test of sperm-cervical mucus interactions involves the penetration of cervical mucus by sperm in vitro. The mucus is placed in a capillar tube, one end of the tube is dipped in semen and penetration and motility of sperm in the mucus column is measured. It can be performed with homologous or donor cervical mucus. Using cervical mucus standardized by estrogen treatment, this test was shown to have good predictive value of fertility. Alternatively to human cervical mucus this test can be performed with commercial midcycle bovine cervical mucus or hyaluronic acid gels. The use of alternative material to human cervical mucus has practical advantages (availability, reproducibility) but may be less informative than human material, due to differences in the nature of the hydrogels. Therefore whenever possible human cervical mucus should be used and for specific male factor detection oestrogen standardized donor mucus should be preferred.

Sperm functional assays

Sperm functional assays have been developed in an attempt to find a good predictive test of male fertility. We will discuss the hemizona assay, the hamster egg penetration assay and the sperm hypo-osmotic swelling assay.

Hemizona assay

The hemizona assay (HZA) measures the binding of capacitated sperm to isolated human zona pellucida. Human oocytes are bisected by micromanipulation, thus allowing for an internally controlled comparison of sperm binding (from patient versus a fertile control) to matching hemizona surfaces. The two matched hemizona of the human oocytes have the advantage of providing functionally equal surfaces allowing a controlled comparison of sperm binding and therefore limiting the amounts of oocytes used. Ethically this assay is acceptable since the microsurgical bisection of the oocyte prevents any inadvertent fertilization. The HZA has been found to be predictive of IVF outcome with positive and negative predictive values of 83% and 95% respectively. The major problem with this assay is the limited availability of human oocytes. Eventually it could be replaced by a standardized kit in which recombinant human zona sperm receptors mimic the natural functional hemizona used now.

Sperm penetration into zona-free hamster oocytes

The zona-free hamster oocyte sperm penetration assay is a heterologous bioassay has originally been developed to test capacitation, acrosome reaction, fusion and sperm chromatin decondensation. Cross-species fertilization is made possible by removing the zona pellucida of hamster oocytes. Using the original test conditions the limiting step is the low incidence of spontaneous acrosome reaction in human sperm populations incubated in vitro, and therefore it has been described as measuring the ability of sperm to undergo acrosome reaction rather than the overall fertilization process. Optimized procedures including acrosome reaction induction by ionophore A23187 are giving good predictive value of IVF and of incidence of pregnancy in the absence of treatment.

Hypo-osmotic swelling of sperm flagella

The hypo-osmotic swelling test (HOS) measures sperm membrane integrity as ability to swell when exposed to hypo-osmotic media. The biologic significance of this test is unclear and its validity to predict IVF fertilization rate is controversial, and it is equivalent to viability staining. The HOS test and correlates with semen analysis data but not with the hamster oocyte penetration test.



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