

Standardized procedures in human semen analysis

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Semen analysis procedures vary between institutions. There have been several attempts to standardize some of the procedures so as to facilitate inter-laboratory comparison of data. This paper describes some of the procedures which can be used quite conveniently in our laboratories. This paper also describes a method for interpreting data obtained from semen analysis.

THE only alternative to assess a man's fertility, other than asking him to sire a child, is to examine his semen. Semen comprises of spermatozoa and other cells suspended in a fluid matrix, the seminal plasma, which acts as a buffering and nutritive medium. A number of characteristics of the semen have been attributed to be of value in assessing its fertilizing potential.

It has been well-recognized that methods for analysing semen and the criteria used to assess the data differ between investigators and laboratories. Recognizing the need for using standardized procedures, the World Health Organization as well as the Indian Council of Medical Research have brought out manuals describing standardized methods for semen analysis. Despite such efforts most laboratories still do not use standardized procedures and consequently inter-laboratory data on semen quality are not comparable. The reason for this state of affairs is either ignorance of the existence of standardized semen analysis procedures or a lack of appreciation of the merits of the various techniques used.

This article reviews some of the standardized procedures used for semen collection, assessing its physical, microscopic and chemical features and the value of these features in evaluating the fertilizing potential of a semen sample.

Collection of semen

Frequent ejaculation as well as prolonged sexual abstinence are known to change the profile of semen. Ideally, samples should be collected at intervals similar to the man's coital frequency. An interval of 3 to 5 days between the last ejaculation and semen collection is recommended because shorter or longer intervals could give a false impression about the normal physiological status of semen quality¹.

Semen should be collected by masturbation into a sterile glass beaker or wide-mouthed jar; plastic con-

tainers should be avoided unless they are proven to be non-spermicidal. Samples should not be exposed to extremes of temperatures and analysis should be carried out within 30 min of collection².

Semen analysis

Physical features of semen

The volume, colour and viscosity of freshly collected semen and the presence or otherwise of a coagulum must be recorded for each sample as these are important indices to semen quality.

Semen volume can be determined to the nearest 0.1 ml by transferring the entire sample into a graduated centrifuge tube which can be categorized as normal, high or low volume (Table 1). Almost 15–20% of men who seek treatment for infertility have either low or high volumes of semen^{3–5}. Secretions from the prostate gland and seminal vesicles, whose functions are androgen-dependent, contribute to the bulk of the seminal fluid. Low semen volume, associated with low levels of circulating androgens, can be treated by administering human chorionic gonadotropin but not those which are not associated with androgen deficiency. High semen volumes may be due to the accessory reproductive glands being hyperactive or due to an extended interval between the last ejaculation and semen sampling. Collection of semen by the split-ejaculate method or mild centrifugation (500 g for 5 min) of semen is sometimes useful for recovering reasonable concentrations of

Table 1. Range of values for routine 10 parameters in the three classes of semen samples

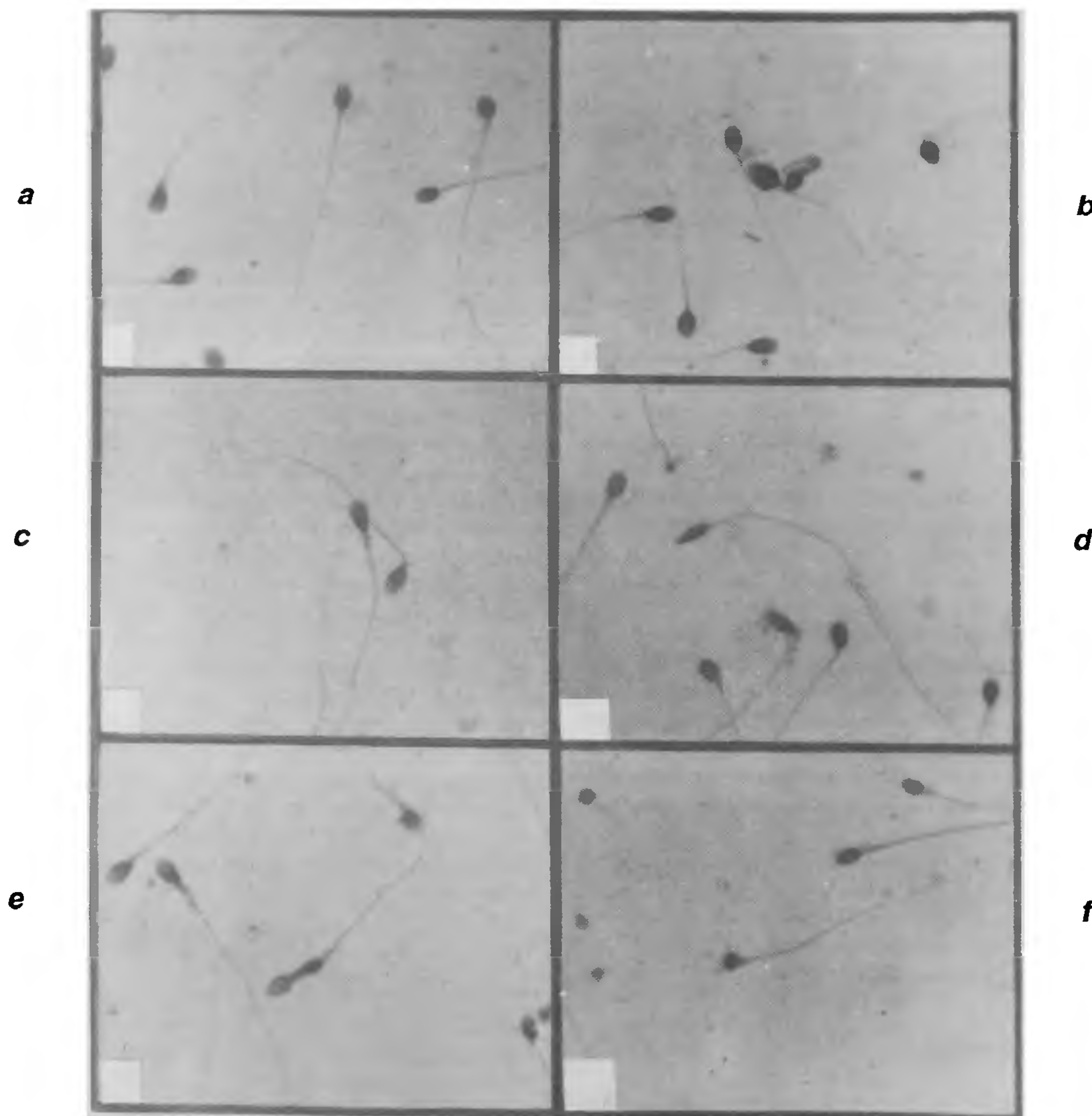
Parameters	Fertile	Subfertile	Infertile
Liquefaction (mts)	20–30	31–45	> 45
Volume (ml)	1.5–4.5	4.6–5.0	> 5.0
		1.4–1.2	< 1.2
Viscosity	Normal	Moderate	High
Amorphous debris	Nil – ++	+++	++++
Agglutination (%)	Nil	1–10	> 10
Motility (%)	> 50	35–49	0–34
Viability (%)	> 60	40–59	< 40
Count (mill/ml)	> 20	10–19	< 10
<i>Morphology</i>			
Normal forms (%)	> 35	30–34	< 30
Headless (%)	< 15	16–20	> 20

spermatozoa for any of the therapeutic insemination methods.

Coagulation of semen is caused by the secretions from the seminal vesicles. Coagulated semen should normally liquefy within 15–20 min after standing the sample in the laboratory; this is due to the action of prostatic secretions on the coagulum. Non-liquefaction indicates

dysfunction of the prostate⁶ and of lack of prostatic proteinases like seminin⁷. Several agents like alevair, trypsin and 5% α -amylase have been used in the laboratory^{8,9} to liquefy coagulated semen.

The strong characteristic odour of normal semen is due to the oxidation of prostatic spermine. Malodorous semen is suggestive of infection.



Figures 1–4. 1, Sperm morphology The photomicrographs have been prepared from seminal fluid specimens stained with Papanicolaou's stain and photographed at a magnification of 1250 \times . The germ cell classification is based on that proposed by Macleod, J., in *The Human Testis*, Plenum (Rosenberg and Paulsen) 1977, p. 481. *a*, Normal spermatozoa (N), Oval-shaped head (3–5 μ m long, 2–3 μ m wide) with regular outline with no other defects. Midpiece less than one third the width of head, and regular in outline (7–8 μ m long) tail is slender uncoiled, with regular outline (40–45 μ m) both midpiece and tail are aligned along the longitudinal axis of the head, *b*, Large oval head (LH), Head size three times larger than normal spermatozoa with regular outline. The mid-piece and tail appear normal, *c*, Pear-shaped head (PH), Heads take the shape of a tear-drop. Tapered end coming to a point just above mid-piece. The mid-piece and tail appear normal, *d*, Tapering head (TH), The head length remains the same but width decreases. Head assumes a 'cigar' shape. The mid-piece and tail are normal, *e*, Amorphous head (AH), The heads having bizarre shape and cannot be classified into any other category of head abnormality, *f*, Round head (RH), The head of the spermatozoa is absolutely round possessing no acrosome and with regular outline.

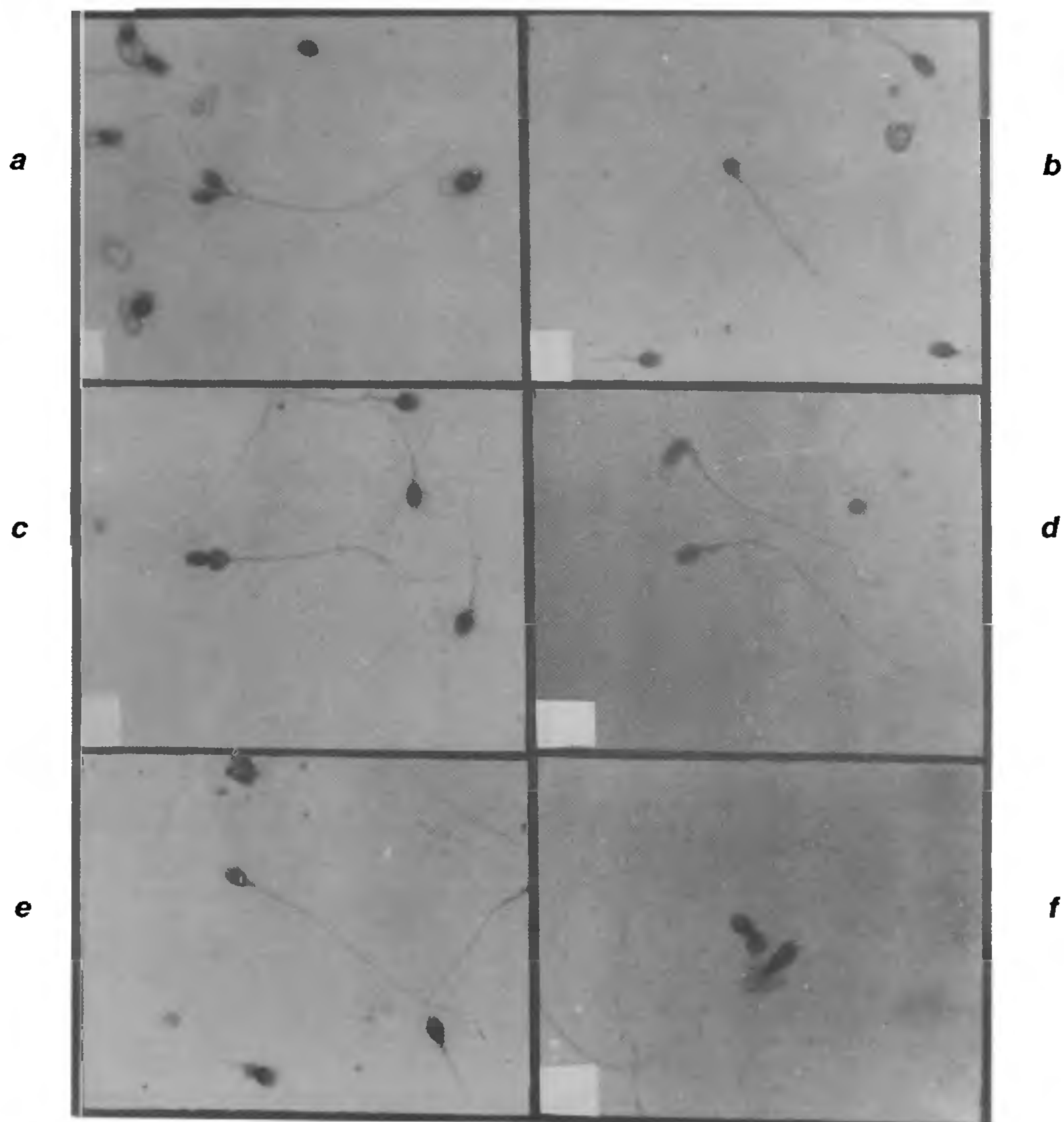


Figure 2. *a*, *Double head (DH)*, Spermatozoa showing two distinct normal heads, it may be of different shapes also. The mid-piece and tail appear thick, *b*, *Small head (SH)*, Spermatozoa show a very tiny round head with irregular outline. The mid-piece and tail appear normal, *c*, *Cytoplasmic droplet (CD)*, The cytoplasmic droplet is seen attached to the mid-piece. The tail appears normal, *d*, *Bent neck (BN)*, The spermatozoa show a clear bent at right angles to the longitudinal axis. The heads and tail appear normal, *e*, *Thinned-out mid-piece*, Spermatozoa showing normal head with midpiece that is thinned out (absent mitochondrial sheath) and continued with normal tail, *f*, *Only heads (H)*, Spermatozoa seen as 'free' or 'loose' heads with little cytoplasm attached.

Semen is normally greyish white. Yellow colour indicates infection and a reddish brown sample is suggestive of internal haemorrhage or the excretion of dyes used in medicinal tablets ingested by the individual. Black or dark grey semen is associated with certain metabolic disorders such as alkaptonuria.

Viscosity of seminal fluid can be determined by using capillaries, calibrated glass tubes or rotational visco-

meters¹⁰⁻¹³, a simple needle and syringe or a 0.1 ml graduated pipette¹⁴. Hyperviscous semen is mostly associated with bacterial infection¹⁵ or presence of antisperm antibodies¹⁶ or increased number of morphologically abnormal forms of spermatozoa and low fertilization rates^{17, 18}. It may be useful to collect semen by the split ejaculate method to obtain good quality spermatozoa for therapeutic insemination from indivi-

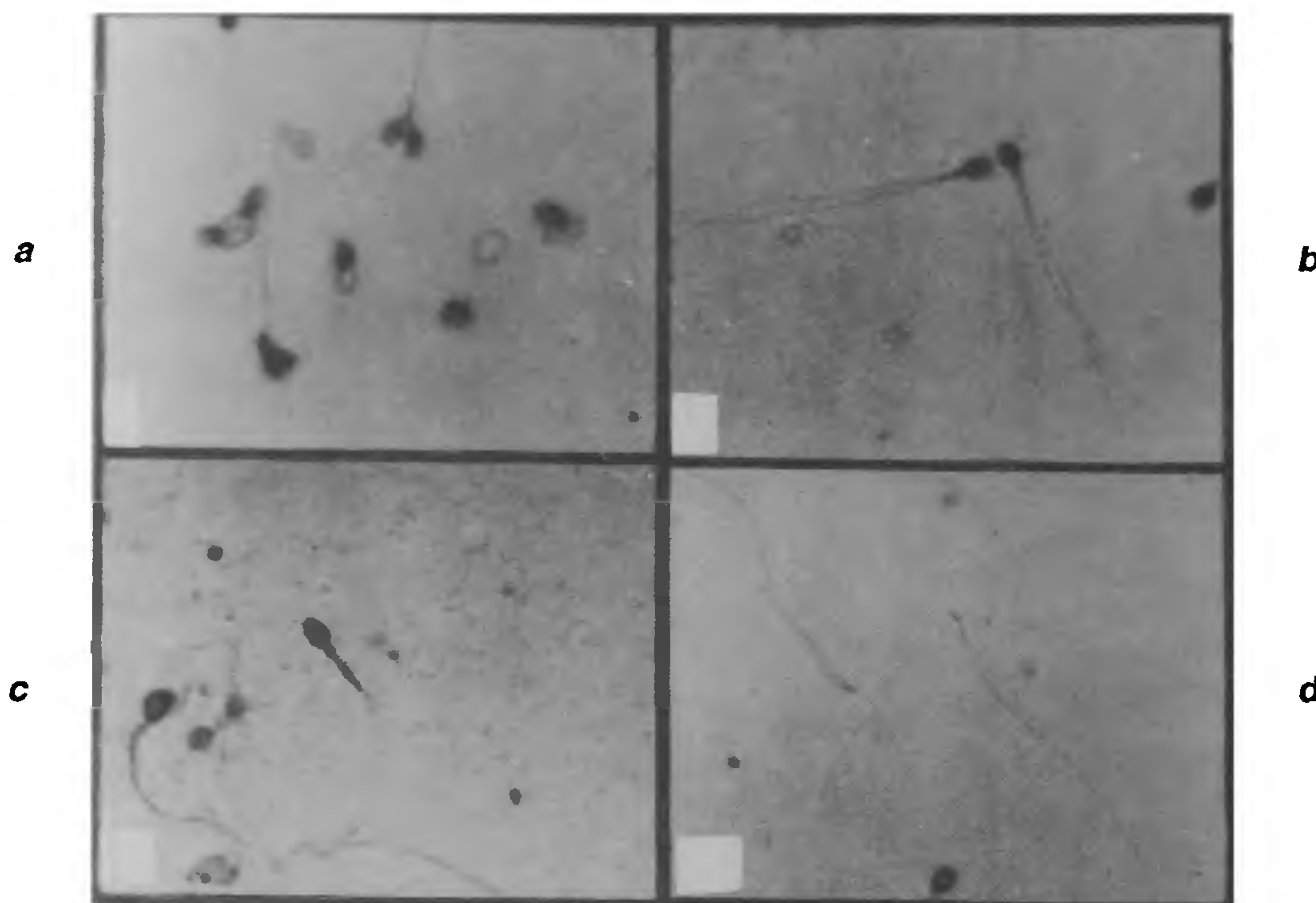


Figure 3. *a*, Coiled tail (CT), The tail is rounded over itself forming a compact coil. Coiling may be seen only at the ends forming loose loop or compact as shown, *b*, Double tail (DT), Spermatozoa show normal head with double tail. The mid-piece appears also to be double. The head is normal in shape, *c*, Stumpy tail (ST), Spermatozoa show normal head with a short thick stumpy tail, *d*, Headless (HL), Spermatozoa actively motile showing only mid-piece and tail. They should be differentiated from spermatozoa with small head (as shown in Figure 2 *b*). A separate count of these headless should be done and expressed as percentage of headless to spermatozoa with head.

duals having highly viscous semen as first portion of the split ejaculate is less viscous and has better sperm count and motility. Sputolysin or bromelain (dithiothreitol in phosphate buffer saline) have been used to reduce viscosity^{19, 20}.

It has been a conventional practice to determine the acidity or alkalinity of semen with a pH paper (6.6–8.0) or a pH meter which gives greater accuracy. There is no good evidence to suggest that pH is an indicator of sperm quality. Osmolality of seminal fluid ranges between 360 and 380 mOsm/kg. Hypoosmolality alters sperm morphology mainly leading to tail defects²¹. The quality of spermatozoa from semen with low osmolality can be improved either by processing semen by any one of the sperm-processing methods or by treating them with heterologous seminal plasma from a normal individual.

Microscopic features of semen

Useful information can be gleaned by light or optical microscopy of a drop of semen evenly spread out on a

glass slide and examining random optical fields using $\times 40$ objective.

Absence of spermatozoa must be confirmed by mildly centrifuging (500 g for 15 min) the sample and examining the pellet smeared on to a microscopic glass slide. The following features must be examined in samples having spermatozoa:

Motility. The conventional grading of sperm-motility into four categories is very subjective and adequate precautions must be taken before such observations are made to increase reliability. An evenly spread smear is likely to give more reliable information than an unevenly spread smear; samples exposed to extreme temperatures following ejaculation can adversely affect sperm-motility. Spermatozoa finally acquire their characteristic type of mature motility during their transit through the epididymis²² and therefore any abnormal or reduced motility observed in the ejaculate could be due to epididymal dysfunction²³ rather than due to intrinsic sperm defects.

Sperm-motility is an important attribute of sperm quality as there is a good correlation between sperm-

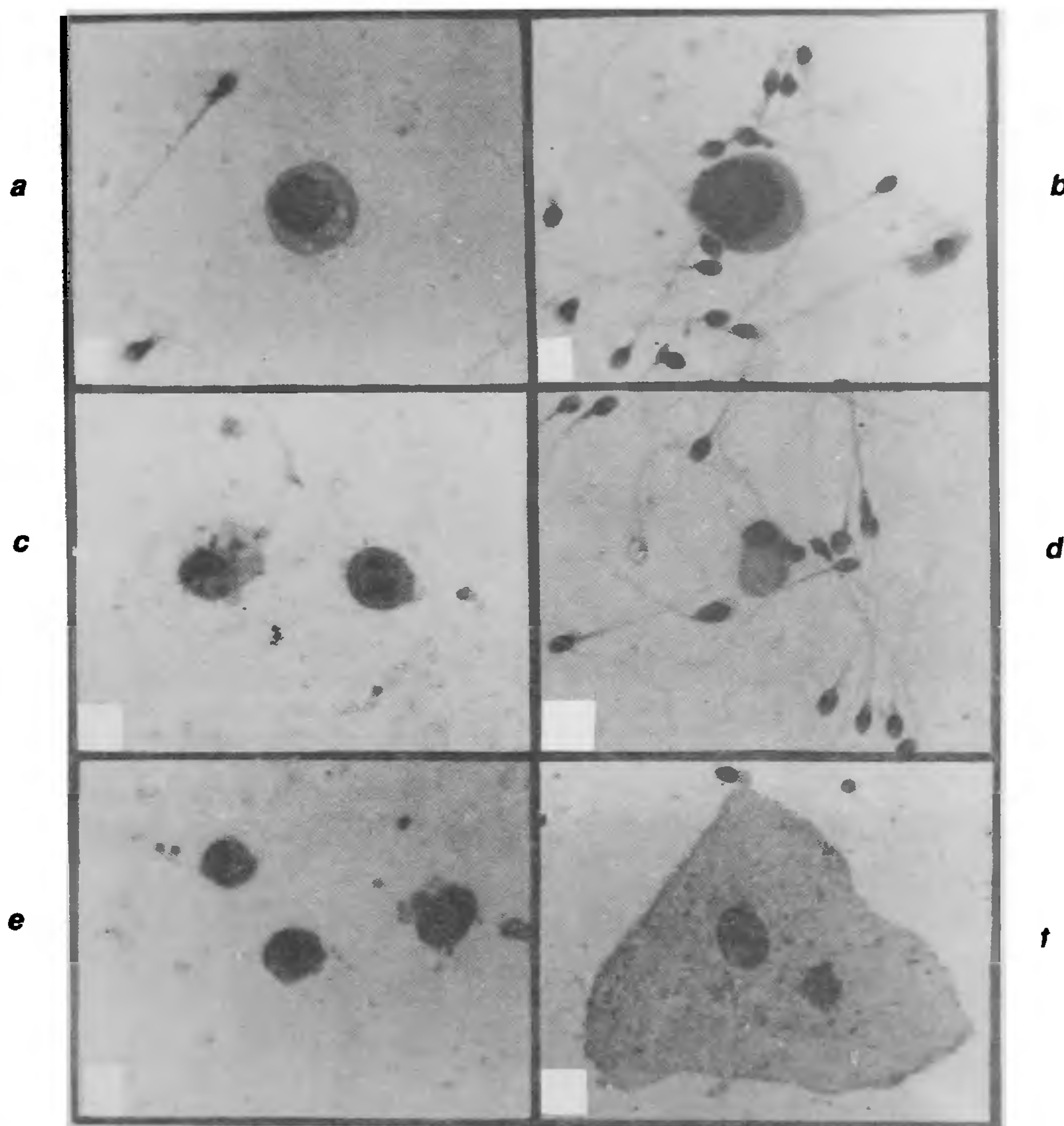


Figure 4. Non-spermatozoal cells and immature germ cells. *a*, *Spermatogonia*, These cells have a large nucleus (6 to 7 μ m) with one or two nucleoli seen resting on the edge of the nucleus; *b*, *Primary spermatocyte*, Cells with large nuclear diameter (8–9 μ m) stained dark violet to purple in a greyish cytoplasm. Chromatin is usually homogenous but occasionally chromatin threads can be seen; *c*, *Secondary spermatocyte*, Cells stain like primary spermatocytes but smaller in diameter. The nucleus is spherical (nuclear diameter 7 μ m); *d*, *Spermatids*, Cells are usually spherical, sometimes small magenta-coloured acrosomal cap can be seen. Several spermatids may share a common cytoplasm; *e*, *Polymorphonuclear leukocytes*, These cells show purple to violet nuclei with pale blue cytoplasm or the cytoplasm may be granular; *f*, *Epithelial cells*, Large polygonal cells, the significance of many epithelial cells (? contamination) is yet to be clearly defined

motility on the one hand and plasma membrane integrity and conception rates on the other^{24–26}. However, merely improving motility by any one of the *in vitro* semen processing methods and the highly motile sperms obtained for intrauterine insemination does not significantly increase conception rates^{27, 28}.

Objective methods for assessing sperm motility such as, time lapse exposure/multiple exposure photomicro-

graphy^{29, 30}, light scattering and turbidity methods^{31–33}, videographic recordings of sperm motility and photon correlation spectroscopy^{34–40} have been experimented upon in recent years.

Sperm density. Sperm density is routinely determined using a haemocytometer and expressed as millions of spermatozoa per ml of semen. Other methods include

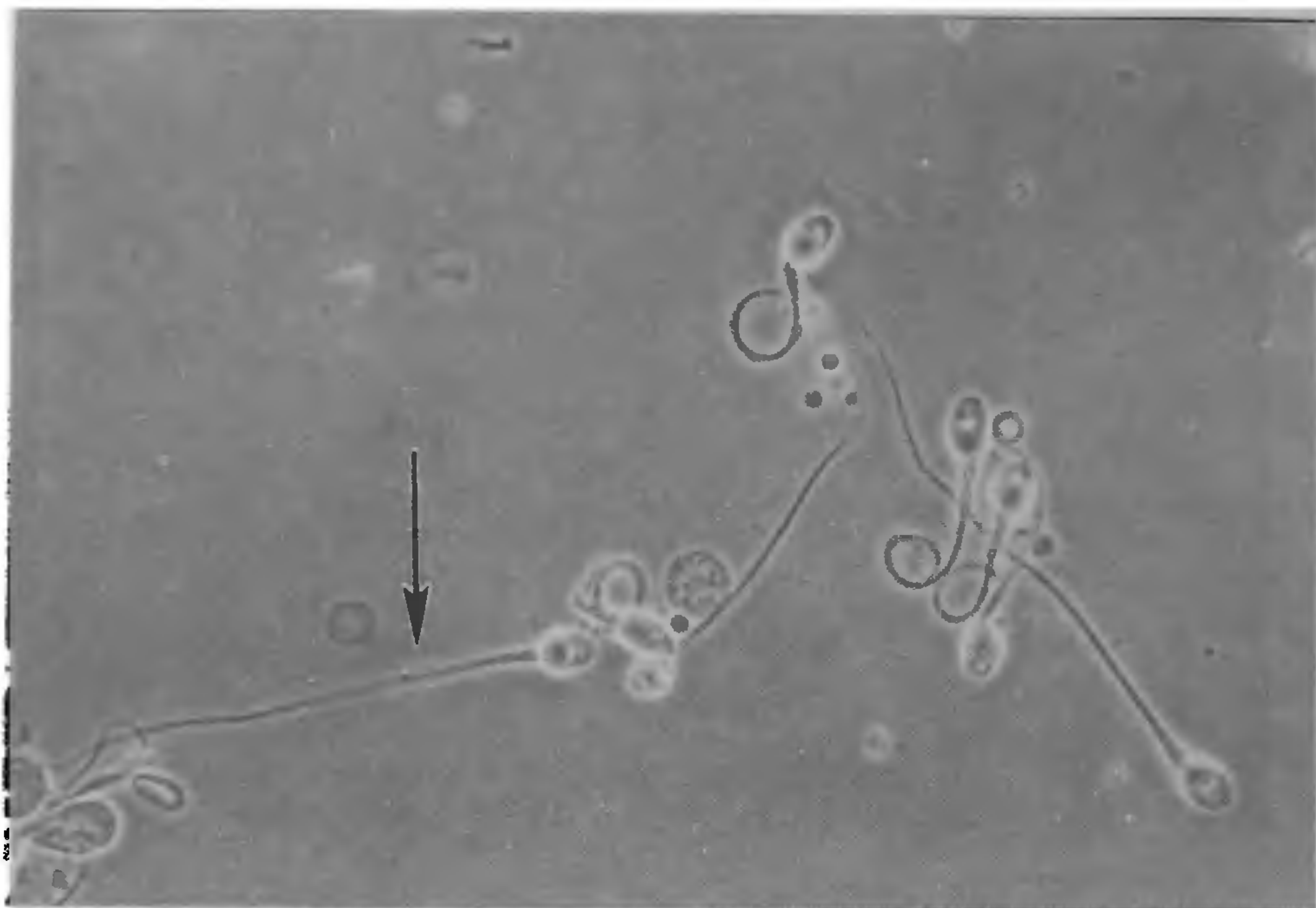


Figure 5. *Hypoosmotic swelling test.* This figure shows the effect of hypoosmotic medium on spermatozoa. Spermatozoa with coiled tail indicate positive test with normal plasma membrane integrity. The spermatozoa with straight tail (↑) indicate negative test ($\times 400$).

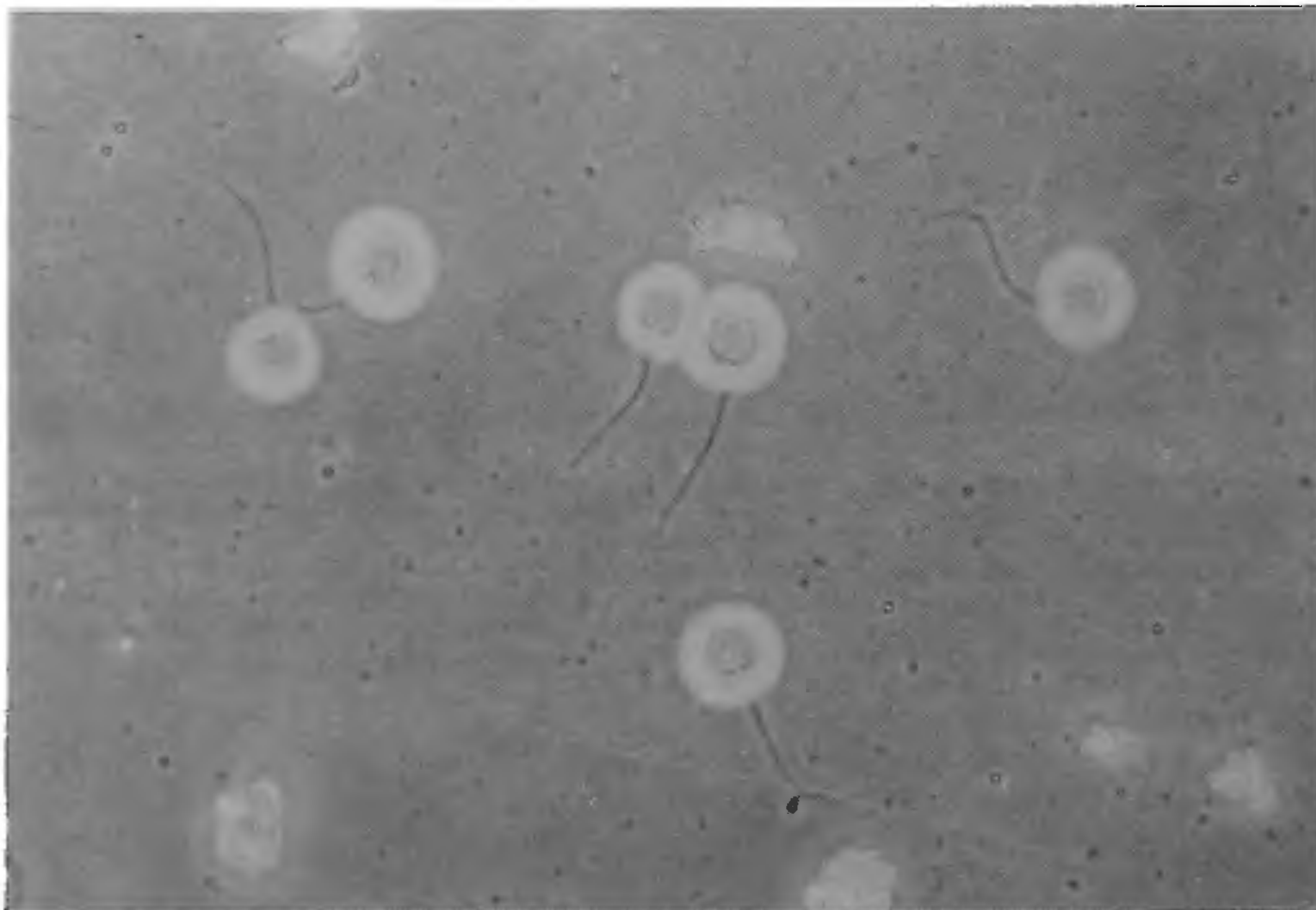


Figure 6. *Test for acrosome intactness.* The picture shows the halo around the head of spermatozoa which is the area of lysis of gelatin ($\times 400$).

the use of Makler chamber, coulter counter and computer-assisted semen analysis systems^{41, 42}. The total sperm count is the total number of spermatozoa in the entire volume of the ejaculate (Table 1). It is important to take a drop of semen from a well-mixed sample and to improve accuracy to take a drop from two different dilutions of semen. Sperm density is known to vary between samples taken from the same individual over a period of time due to a variety of reasons⁴³. Since pregnancies have been reported in individuals with persistently low sperm counts (< 20 mill/ml), sperm density is not necessarily a good indicator of a man's fertility status.

Sperm morphology. The relative distribution of the different morphological types of spermatozoa present in a sample provides the most significant clue to discriminate between fertile and infertile samples⁴⁴⁻⁴⁶. Air-dried smears of semen stained with Papanicolaou's or Shorr's stain are best for identifying the different morphological types of spermatozoa. Other techniques include morphometry⁴⁷ and video overlay⁴⁸. Approximately 200 spermatozoa, distributed randomly, should be observed in a stained microscopic preparation and classified into the different types^{20, 46, 49}. Photomicrographs of the different sperm types may be used as reference standards to reduce interpersonnel, observational differences (Figures 1-4). Morphological abnormalities can be categorized into those related to the

head, midpiece or the tail; headless spermatozoa need to be put into a separate category. A high incidence (> 20%) of headless spermatozoa, usually referred to as 'pin heads', is indicative of male infertility⁵⁰. Sperm movement is reportedly different between morphologically normal and abnormal spermatozoa⁴⁸.

The use of scanning and transmission electron microscopy^{51, 52} for evaluation of sperm morphology is not practical as a routine method of analysis.

Non-spermatozoal cells. These include immature germ cells, leucocytes and macrophages. Exfoliated prostatic cells have been occasionally described in semen⁵³ but their role, if any, is unknown. The use of specific monoclonal antibodies coupled with immuno-histochemistry or electron microscopy aid in the accurate identification of the cell type⁵⁴. Bacteria, fungi, protozoans and viruses have deleterious effects on the motility and other characteristics of sperm and may also cause infertility^{15, 55-57}.

Particulate debris. These would appear as an amorphous material in fresh, wet smears of semen and can be graded subjectively on 1-3 scale as nil, mild to moderate and severe. Presence of amorphous material in semen is a good indicator of infection and the sample must be subjected to further culture¹⁵.

Agglutination. Head to head, tail to tail or mixed types of agglutination is indicative of presence of sperm



Figure 7. Nuclear chromatin decondensation test. The figure shows swollen decondensed heads (↑↑) and head which has failed to decondense (^ ^) (× 400)

antibodies in the seminal plasma and merits further examination of the semen using specific immunological diagnostic methods.

Biochemistry

Seminal biochemistry is an indicator of the functional status of the accessory reproductive glands. Zinc, citric acid and acid phosphatase are estimated to determine the functional status of the prostate gland^{58, 59} while fructose levels are indicative of seminal vesicle function. Fructose levels are determined by the resorcinol method⁶⁰ or the more reliable enzymatic technique⁶¹. Absence of fructose in semen is indicative of ejaculatory duct obstruction or congenital absence of vasa deferentia and/or seminal vesicles. Glyceryl phosphoryl choline (GPC), carnitine, neutral α -glucosidase have all been associated with epididymal function^{62, 63}. Concentrations of these substances in semen are variable due to a number of reasons and therefore very little purpose is served by determining the concentrations of the chemicals in the semen.

Sperm function analysis

None of the physical or biochemical features of semen described above can unequivocally establish the fertilizing potential of a semen sample. A number of sperm-

function tests need to be applied to aid in determining the fertility status of a sample.

Zona-free hamster oocyte penetration test. This test⁶⁴ is aimed to establish the sperm's capability to undergo capacitation and acrosome reaction and the nuclear chromatin to decondense. The results of this test are variable and inconsistent, time-consuming, expensive and requires special facilities and skills⁶⁴⁻⁶⁶. There is no consensus on the value of this test as its results do not always correlate well with *in vitro* fertilization or *in vivo* fertility⁶⁷⁻⁷⁰.

Plasma membrane integrity. The integrity of plasma membrane of the spermatozoa is measured by two methods. The first is to subject spermatozoa to vital staining⁷¹ and determining the incidence of unstained, vital sperms versus stained, dead sperms. This test is based on the premise that the living cell membrane acts as a barrier to the stain solution. The second test is the hypoosmotic swelling tests (HOS-test)⁷² which is based on the premise that when viable sperms are incubated in hypoosmotic medium, there is an influx of fluids resulting in the curling up of the tail and bulging of the sperm membrane which can be identified under phase contrast microscope (Figure 5). The first test determines the structural integrity of the sperm whereas the HOS-test establishes the physiological integrity of the plasma membrane⁷³. The integrity of plasma membrane is compromised in semen samples infected with bacteria or *Trichomonas*^{15, 55}.

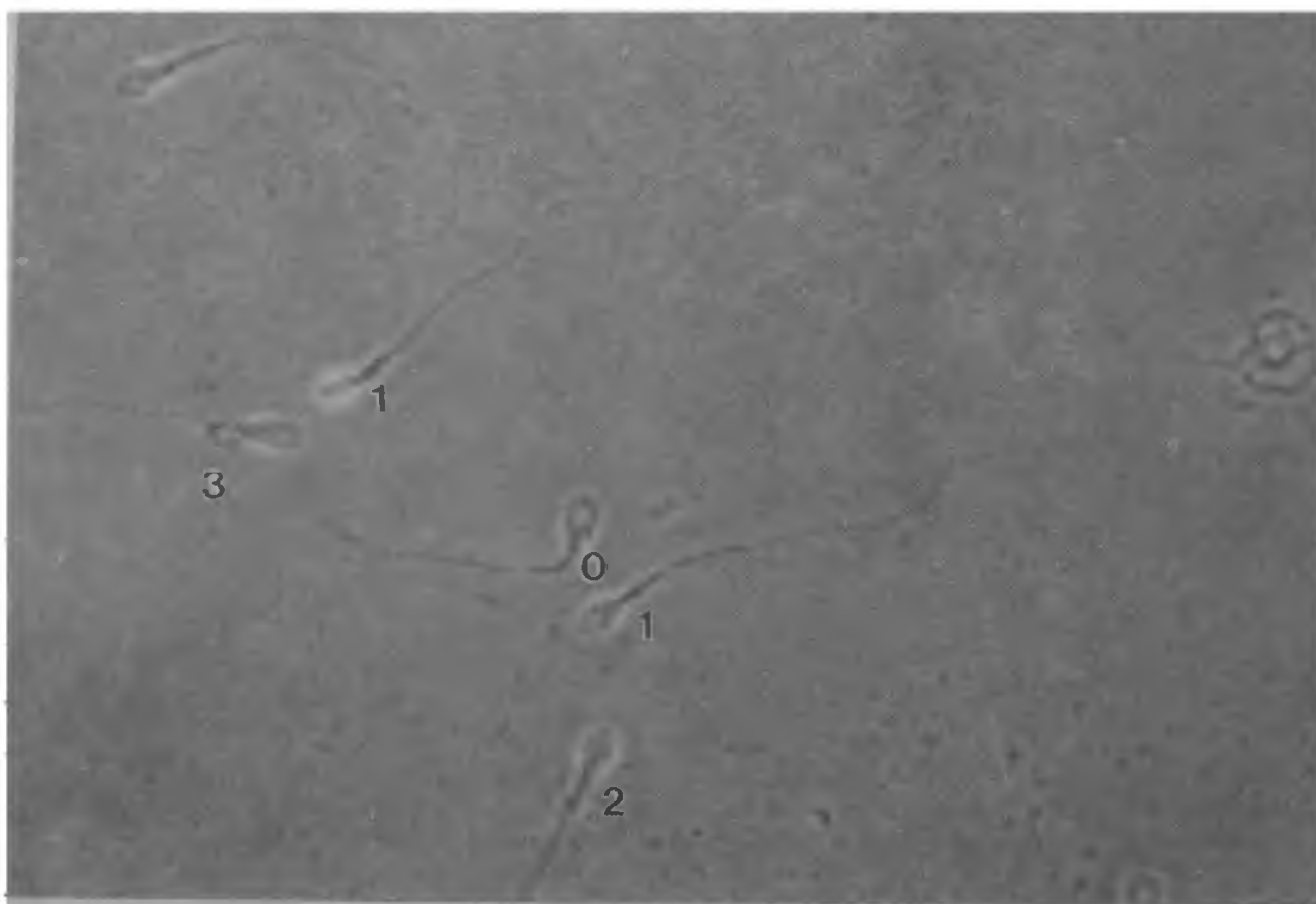


Figure 8. Sperm mitochondrial activity index The picture shows the various grades of dye (NBT) deposit along the mid-piece (classified as nil, standard-1, substandard-2, and low-3 ($\times 1250$))

Acrosomal intactness. Acrosomal enzymes facilitate penetration of the oocyte by spermatozoa. Acrosomal status can be assessed by light, electron and fluorescence microscopy or spectrophotometric determination of acrosin content using mono- and polyclonal antibodies against acrosomal antigens⁷⁴⁻⁷⁶. Acrosin is stated to be a good indicator of a successful outcome of *in vitro* fertilization⁷⁷. A simple inexpensive method to study acrosome status is the gelatin slide test. This test measures the proteolytic activity of acrosin using a gelatin substrate⁷⁸ (Figure 6).

Nuclear chromatin integrity. Flow cytometry, the acridine orange staining, *in vitro* decondensation of nuclear chromatin are some of the methods available to determine the integrity of nuclear chromatin⁷⁹⁻⁸³ (Figure 7).

Sperm-mitochondrial activity index. Sperm-mitochondrial enzyme can be cytochemically visualized, categorized and their index estimated⁸⁴ (Figure 8). This test is useful to differentiate between asthenozoic spermatozoa with mitochondrial inadequacy from those with tail defects.

Scoring of sperm analysis data

As mentioned earlier, none of the features of the semen described above can unequivocally categorize a sample to be fertile or infertile. All these features are extremely variable between individuals as well as between samples. We have attempted to introduce a very arbitrary form of scoring the data to enable a ready categorization of semen into one of three groups, viz. fertile, infertile and sub-fertile on the basis of values obtained for about 10 features of the semen which have been chosen on the basis of their functional importance. A score of 2 is given to each of these 10 features if the value for each of them is within 'normal' limits as determined for our population and whose semen has been proven to be fertile by *in vitro* fertilization. Thus the range for a fertile sample lies between 15 and 20, the score for a sub-fertile sample lies between 10 and 14 and infertile sample invariably has a score of <10. This arbitrary categorization helps in picking out sub-fertile samples, whose quality could be improved by *in vitro* semen-processing methods. Individuals having a score characteristic of an infertile sample need medical attention.

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