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Sperm preparation for assisted conception

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Human semen contains decapacitation factors from which the spermatozoa must be separated in order to proceed to the stages of capacitation followed by hyperactivation and the acrosome reaction which then enables zona binding and fertilization. Following coitus, these effects are achieved on spermatozoa which travel from the ejaculate into favourable fertile cervical mucus. In assisted reproduction, the various spermatozoal processes enabling fertilization must be achieved by *in vitro* techniques. Relatively simple successful methods have been developed for semen preparation from normospermic men with normal sperm function, but many cases of infertility are associated with abnormal semen parameters or abnormal sperm function. Such cases require rather specialized semen preparation techniques.

Not only must spermatozoa be separated from seminal plasma to achieve fertilizing capacity, but the process must be achieved rapidly, preferably within 30 min. It has been shown that longer exposures to seminal plasma *in vitro* can permanently diminish the fertilizing capacity of spermatozoa. For this reason as well as sperm sensitivity to temperature variations, seminal ejaculates should be collected in the immediate vicinity of the laboratory undertaking the preparation procedure for assisted reproduction effective sperm preparation procedures should also remove dead cells, debris, micro-organisms, non-spermatozoal cells (especially inflammatory cells), sperm precursors, immotile and most morphologically abnormal spermatozoa, as well as prostaglandins, other spasmogens and lytic enzymes carried within the plasma.

The range of clinical procedures requiring sperm preparation include intrauterine insemination (IUI), donor insemination, gamete intrafallopian transfer (GIFT) and *in vitro* fertilization (IVF) with uterine transfer of embryos (ET) or tubal transfer of either pronuclear stage oocytes (PROST) or cleaving embryo stages (TEST). The latter procedures are sometimes designated as zygote intrafallopian transfer (ZIFT). Other less well-established clinical procedures which require

sperm preparations include direct intraperitoneal insemination (DIPI) and either peritoneal oocyte–sperm transfer (POST) or transcervical sperm–egg transfer (T-SET).

In clinical practice, sperm preparation techniques were originally developed for IVF where the underlying infertility problem was due to fallopian tube obstruction. The sperm preparation techniques of 'washing' with either over-layering of culture media or placement of the sperm pellet under culture medium (underlayering) followed by spermatozoal swim up and harvest, gave suitable 'sperm preps' for fertilization. However, as male factor infertility and other complex cases were included into the assisted reproduction programmes, these techniques were found to be inappropriate for many cases and often associated with failed fertilization in severe forms of oligo/asthenozoospermia. A variety of other sperm prep techniques have improved the prognosis for such cases and include the use of discontinuous Percoll gradients and sedimentation techniques, reducing or excluding centrifugation procedures when the spermatozoa are considered to be fragile.

Further specialized sperm preps have evolved to cope with specific conditions such as micromanipulation, microinsemination and spermatozoal microinjection; the presence of antispermatozoal antibodies; micro-epididymal sperm aspiration (MESA); and specific spermatozoal dysfunctions. Delicate preparation techniques are required along with substances designed to improve motility parameters, to induce the acrosome reaction or to suppress reactive oxygen species formation.

Swim-up procedures

An overlay technique which has proven suitable for normospermic cases with normal sperm function is schematically described in Fig. 1. It has also proved suitable for moderate oligozoospermic cases but only occasionally for severe cases, by adjustment of the final sperm concentration (Yovich & Stanger, 1984). The aim is to achieve between 50 000 to 100 000 motile spermatozoa as a 'clean' preparation, i.e. devoid of leukocytes, sperm precursors and immotile spermatozoa. Sometimes, larger numbers can be inseminated in IVF or GIFT to compensate for mild to moderate oligozoospermic cases with sperm dysfunction (Matson *et al.*, 1987). In IVF procedures, fertilization rates should exceed 70% of preovulatory oocytes inseminated. For IUI, at least 2 million spermatozoa, and usually 5–10 million where available, should be prepared in 100–200 μl for insemination.

Overlay techniques of sperm prep have been criticized because the centrifugation process may pack the useful spermatozoa into a tight pellet along with all the deleterious factors, including reactive oxygen species from immotile spermatozoa (Aitken & Clarkson, 1988), which are destined to be removed after the two-stage

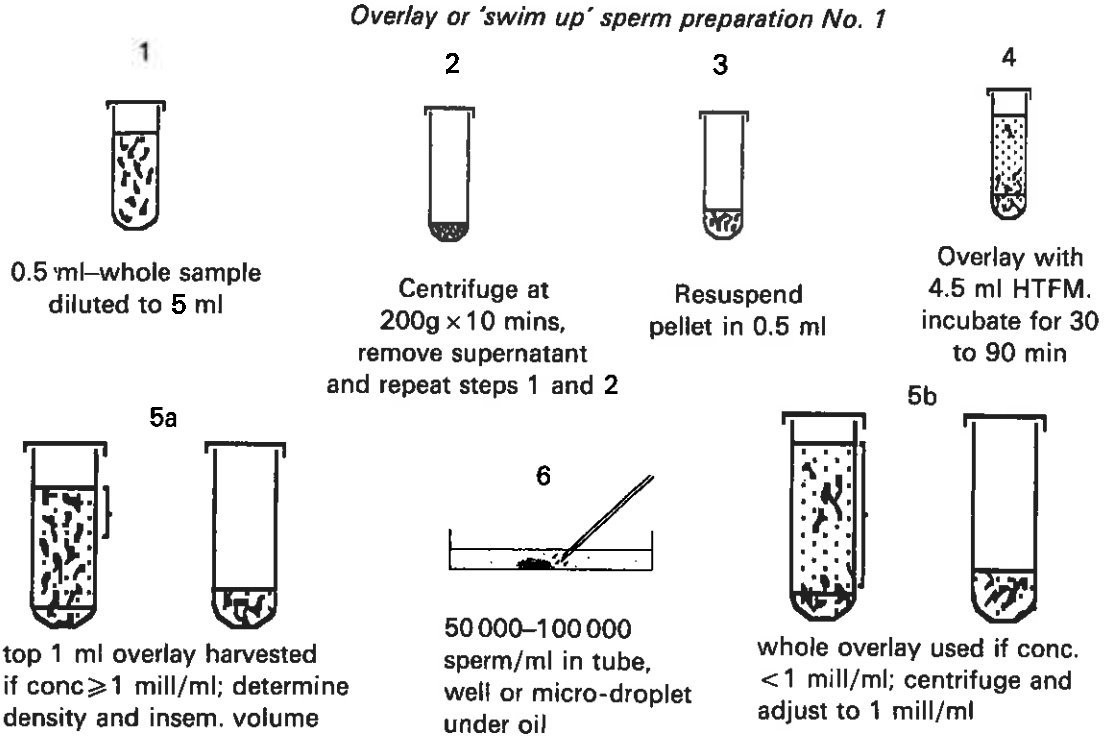

Fig. 1. Sperm preparation technique using washing process followed by centrifugation and overlayering of medium enabling 'sperm rise'. It is important not to exceed 200 g at centrifugation and to avoid tight sperm pellets. The loose pellets are fully resuspended in 0.5 ml medium before overlayering.

wash and centrifugation processes. However, this is not a problem in practice if centrifugation forces do not exceed 200 g; tight pellets are not created; and the loose pellets are fully resuspended in 0.5 ml of sperm prep medium. To avoid these potential problems, the most recent edition of the WHO laboratory manual (WHO, 1992) advises a preliminary swim up of overlayered semen to harvest a relatively clean motile fraction before the washing/centrifugation steps (Fig. 2).

A wide range of culture media appear to be suitable for sperm preps. In the author's experience HTFM (Table 1) or other modified Tyrode's solutions (Yovich & Grudzinskas, 1990) and supplemented Earle's medium (as described in Appendix XXIV of the third edition of the WHO manual (WHO, 1992)) have proved suitable. Culture media are usually supplemented with deactivated serum collected from the female partner after excluding the presence of antispermatozoal antibodies which may inhibit fertilization (Yovich *et al.*, 1984). Alternative protein sources include fetal cord serum, pooled human serum albumin and bovine serum albumin. The latter two are sometimes used in commercial media but serum-transmitted infections remain a serious potential consideration. One pooled human albumin source is Albuminar-20 which has been associated with satisfactory IVF results (Staessen *et al.*, 1990).

Table 1. *Human tubal fluid medium (HTFM)*

Component	Strength
NaCl	101.5 mM
KCl	4.69 mM
$MgSO_4 \cdot 7H_2O$	0.2 mM
KH_2PO_4	0.37 mM
Na lactate	21.4 mM
Glucose	2.78 mM
Penicillin	100 IU/ml
Streptomycin	50 g/ml
$NaHCO_3$	25 mM
Na pyruvate	0.33 mM
$CaCl_2 \cdot 2H_2O$	2.04 mM
Phenol red	0.001%

Osmolality is adjusted to 280 mOsmol and pH to 7.5 for sperm preps. See text reference for full preparation details.

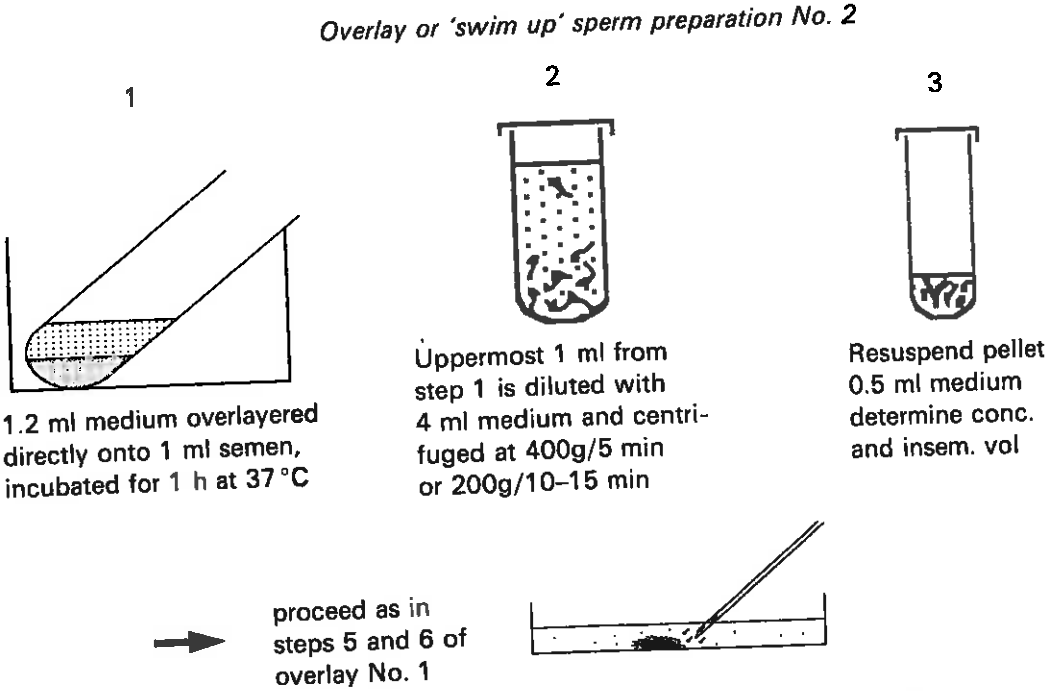

Fig. 2. An overlayering technique of sperm preparation which avoids centrifugation of diluted semen. The first step ensures that the majority of immotile sperm, other cells and debris are left behind when the sperm is subsequently washed, centrifuged and overlayered again. The method is intended to reduce reactive oxygen species from the sperm prep.

Individual sperm preparations

A number of variables within semen are known to correlate with poor fertilization rates and require individualized sperm preps.

Oligo/asthenozoospermia

This term describes semen with progressive motile numbers of spermatozoa <10 million/ml. There is a steadily worsening state such that it is difficult to obtain fertilization with numbers <1 million/ml. However, there is no absolute cut-off, and successful pregnancies have occasionally been generated from sedimentation techniques with motile numbers as low as 200 000/ml.

Teratospermia

Until recently the measurement of single sperm parameters has not shown high predictive values for fertilization *in vitro* although triple sperm defects (i.e. oligo/astheno/teratospermia) have tended to fare poorly. However, Kruger's strict morphology classification (Kruger *et al.*, 1986) does appear to be relevant, i.e. $>86\%$ abnormal forms including those with retained cytoplasmic remnants are associated with a poor fertilization rate. The revised WHO manual also embraces a more strict classification of spermatozoal abnormalities, particularly to include all neck and midpiece defects, and retained cytoplasmic droplets. This may provide a morphological correlation with two biochemical measures of sperm dysfunction within semen and which probably reflect the failure of sperm to shed cytoplasm completely, i.e:

(i) creatine phosphokinase (Huszar, Vigue & Morshedi, 1992)
(ii) reactive oxygen species (Aitken & West, 1990)

Leukocytospermia

Accurate assessment of the number of leukocytes in semen is very important, and detection methods have improved although the popular histological and biochemical methods detect fewer leukocytes than do techniques using monoclonal antibodies. A threshold normal level is difficult to define and appears to be up to 2 million/ml (WHO cut-off is 1 million per ml). Leukocytes will be most relevant if sperm have been exposed to them for a prolonged period and there are associated raised levels of reactive oxygen species (Aitken *et al.*, 1992).

Computer-defined motility dysfunction

After sperm preparation, fertilizing spermatozoa will exhibit more than 10% displaying hyperactivated motility patterns on computer assisted semen analysis (CASA). Hyperactivation is CASA-defined as spermatozoa with high curvilinear velocity, low linearity, and high amplitude of lateral head displacement (Burkman, 1990) along with high beat cross-frequency.

Acrosome reaction

After sperm preparation, at least 10% of sperm (for 85% specificity and 82% negative predictive value) should demonstrate a complete acrosome reaction following ionophore challenge (ARIC). The ARIC score is now used as the main guiding indicator for the use of enhancers of sperm motility such as pentoxifylline and 2-deoxyadenosine (Yovich, Edirisinghe & Yovich, 1994).

Antispermatozoal antibodies

Autoantibodies to spermatozoa will reduce the fertilization rate in direct proportion with the seminal or spermatozoal antibody titre. Special procedures are required to separate out free-swimming motile spermatozoa which may then lead to normal fertilization.

Reactive oxygen species

The superoxide anion, along with the hydroxyl radical and the dismutase-degenerated product hydrogen peroxide, constitute the reactive oxygen species. Their presence in semen may result from leukocytes secondary to genital tract infection, but may also be leukocyte independent. These reactive oxygen species probably cause peroxidation of unsaturated fatty acids in the acrosomal membranes. The ensuing damage may cause the plasma membrane to lose its responsiveness to the calcium influx signal which triggers the acrosome reaction. Sperm preps containing antioxidants such as pentoxifylline, may suppress the formation of reactive oxygen species (Gavella, Lipovac & Marotti, 1991; Yovich, 1993).

Unexplained sperm dysfunctions

Despite seemingly normal parameters, some sperm will persistently fail to fertilize eggs. This situation may be evaluated in a zona binding study, by hamster oocyte

penetration (after induction of the acrosome reaction); or by electron microscopic study of the sperm. After exclusion of genetic disorders microinjection procedures will usually be required. Ethical considerations will also be required when undertaking fertilization with structurally abnormal spermatozoa such as that associated with Kartagener's syndrome (immotile cilia; absent dynein arms) and round-headed sperm disorders.

Specialized semen preparations

The principles of sperm preparation for specialized cases are to produce a seminal plasma-free suspension of highly motile sperm (ranging from progressive to hyperactivated motility) with mostly normal morphology, free from seminal debris and other cells. Depending upon the clinical circumstances and the fertilization culture system, the actual number of sperm required will range from as few as 250 up to as many as 150 000. The larger number is required for oocytes cultured in tubes containing 1 ml of medium; the smaller number for oocytes cultures in straws with as little as 5 μl medium. In each case the sperm concentration is similar. Most units dealing with large numbers of male-factor cases will prefer to culture in microdroplets of variable sizes under paraffin oil. Sperm microinjection procedures such as subzonal insemination and intracytoplasmic sperm injection entail the use of one to five sperm per oocyte, so that theoretically even fewer spermatozoa are required in the harvest for such cases. In normospermic cases good fertilization rates can be achieved with as few as 20 000 spermatozoa/ml in the insemination droplet. However in cases with dysfunctional sperm, particularly where the acrosome reaction rate is known to be very low, one may aim for higher insemination concentrations in order to compensate for the reduced proportion of fertilizing sperm present.

Viscous semen

Poorly liquefied and viscous semen may be an effect of infrequent ejaculation or a persisting unexplained feature in some men. Such samples can be difficult to handle and are best managed by adding culture medium to the semen and frequently pipetting the sample with an undrawn pipette. Severely viscous samples can be treated with enzymic digestion using α-amylase or chymotrypsin for 15–30 min. The common practice of drawing viscous semen through a fine-bore needle causes spermatozoal damage as well as a low yield of motile spermatozoa and should be discouraged. Centrifugation through discontinuous Percoll columns can be very effective (see below) but higher centrifugation forces may be required.

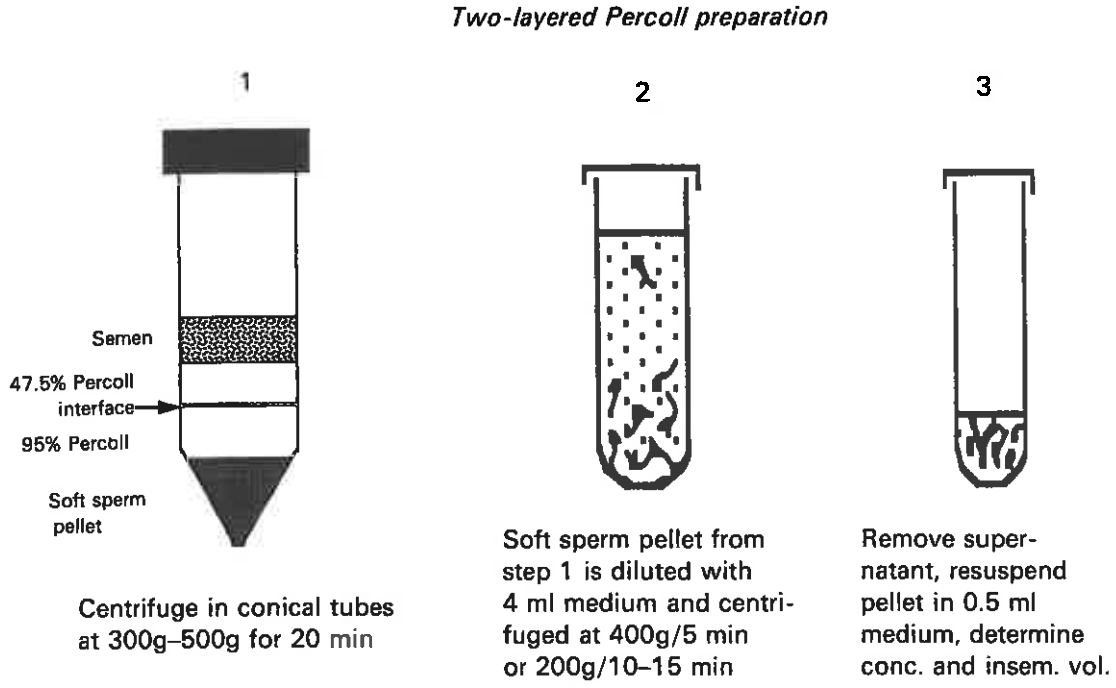

Fig. 3. Neat semen is centrifuged through a two-layered (discontinuous) Percoll column. The Percoll layers are prepared in culture medium and a soft sperm pellet containing mostly motile spermatozoa appears at the bottom of the conical tube, whence it is removed by pipette for subsequent washing, mainly to remove Percoll fragments. Most of the cellular debris and immotile sperm becomes trapped at the interface between the Percoll layers.

Discontinuous Percoll columns

Although many assisted reproduction units have now converted to two-layered Percoll for their routine preparations, the experience at PIVET has not been trouble-free, despite beginning to explore the use of Percoll from 1983. On several occasions fertilization problems have been traced back to Percoll, despite satisfactory results in the quality control system (sperm motility survival measured at 48 and 72 hours). Both IUI and IVF results have been more consistent since introducing routine filtration and re-sterilization of the Percoll in a dry heat oven (120 °C for 8 hours). Two-layered Percoll (e.g. 47.5%:95%) (Mortimer, 1990) or 40%:80% (WHO, 1992)) is used on moderate oligo/asthenospermic cases particularly where there is increased abnormal sperm (e.g. >50%), high viscosity semen, raised leukocytes or high debris content (Fig. 3). Three-layered Percoll (mini-Percoll 50%:70%:95%) (Ord *et al.*, 1990) is used for MESA cases if the sperm is contaminated with blood cells and degenerate sperm (Fig. 4). Sometimes a single layer (70%) is used for preliminary cleanup of samples with a very high content of debris and degenerate sperm prior to sedimentation (see below).

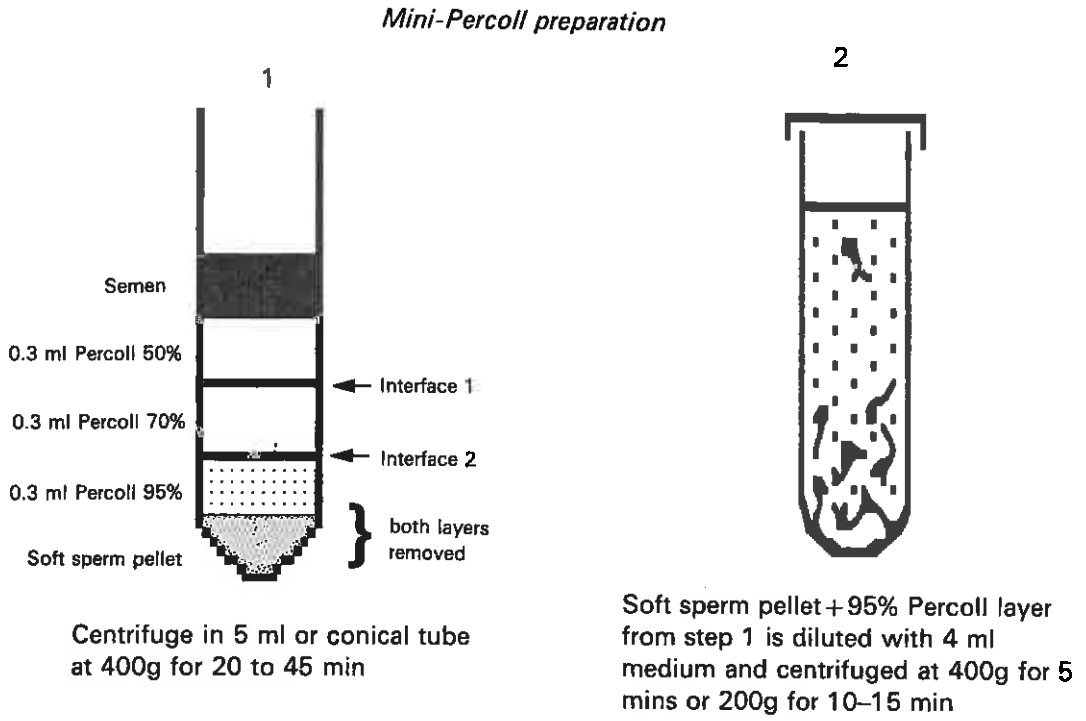

Fig. 4. The mini-Percoll technique involve a three-layered discontinuous column designed especially for small volume MESA aspirates. This entails small volumes in small tubes but the method can be adapted for other, larger volume situations where there is much cellular and particulate debris to be extracted. Such material will appear at the two interfaces and motile spermatozoa are removed from the bottom along with most of the 95% fraction where some motile spermatozoa will be suspended.

Sedimentation and migration techniques

Sedimentation methods (Cohen *et al.*, 1985) are applied for severely oligo/asthenozoospermic cases, i.e. <5 million motile sperm/ml. Depending upon the severity, the following order may be applied:

(i) Single wash to remove most plasma (can be wash/spin or single layer Percoll 70%/spin), followed by sedimentation in Nunc four-well dish. Centrifugation forces should not exceed 200 g in poor quality sperm preps;
(ii) Overlayering of culture medium in tilted round-bottomed 15 ml Falcon tubes, frequent gentle agitation and allow to sediment, collecting small volumes of motile sperm occasionally over 2–2½ hours. Several tubes with a high medium/semen ratio are required (as in the first step of Fig. 2). However, no centrifugation is involved;
(iii) True sedimentation and migration techniques in Tea-Jondet glass tubes (Fig. 5) (Tea, Jondet & Scholler, 1983). This method is ideal for the worst semen samples. Again, no centrifugation is involved.

Sedimentation technique with Tea-Jondet tube

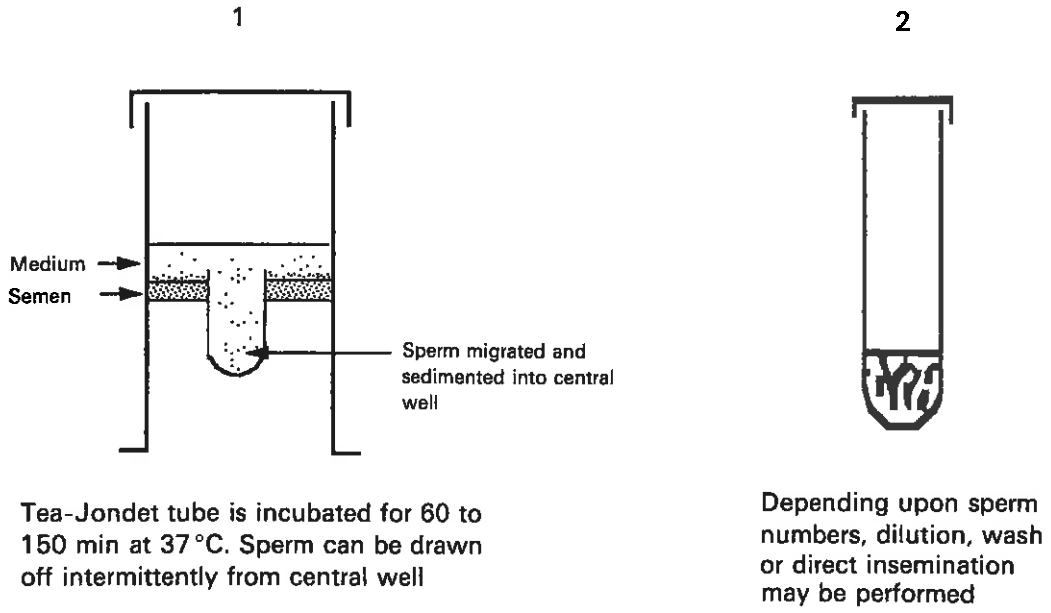

Fig. 5. Tea–Jondet tubes comprise a small tube creating a well within a larger tube. Plastic models are commercially available or they can be hand made from borosilicate glass. They are particularly suited to high debris, oligo/asthenozoospermic cases and the central well can be aspirated intermittently over 2 to 3 hours if required until sufficient spermatozoa are recovered.

Antispermatozoal auto-antibodies

Useful samples can be achieved, even with high antibody levels. by receiving the semen sample immediately after it has been ejaculated directly into culture medium and submitting it to Immunobead separation (Grundy *et al.*, 1992). In this situation, it is imperative that the sperm collection facility is adjacent to the semenology laboratory, e.g. where semen samples can be received through a double door hatchway system with an alerting buzzer triggered by the patient.

Other sperm separation methods

A variety of other sperm separation methods have been reported but have not demonstrated any advantages over the aforementioned techniques and have not gained wide popularity. These include:

(i) the Sperm Select System which has the benefit of simplicity and can generate a 'clean' sperm prep, but with a lower yield of motile spermatozoa;
(ii) transmembrane migration where sperm travel into the culture medium across a nucleopore membrane filter. Again, yields are very low;

(iii) adherence techniques involving glass wool columns or glass beads which cause immotile spermatozoa to adhere to the glass surfaces whilst motile spermatozoa swim through the system; and
(iv) multilayered columns of albumin with varying densities have been applied for sex preselection with some reported success (Beernink, Dmowski & Ericsson, 1993).

Pentoxifylline

We have described the *in vitro* use of Pentoxifylline (PF: 1-9{5-oxohexyl}-3,7-dimethylxanthine), a phosphodiesterase inhibitor, to enhance spermatozoal motility and improve the fertilization rate of oocytes in male factor infertility treated by assisted reproduction. PF 1 mg/ml (3.6 mM) is incubated with the washed sperm preparation after sperm rise or sedimentation and subsequently eluted out prior to insemination of the partner's oocytes (Fig. 6) (Yovich *et al.*, 1988, 1990). The actions and applications of PF in assisted reproduction have recently been reviewed (Yovich, 1993) and can be summarized as follows:

(i) The acrosome reaction can be improved by PF in dysfunctional semen samples (Tesarik & Mendoza, 1993). The optimum indication is where the ARIC score is $<10\%$ but shows improvement with PF as this is usually the relevant end-point to be achieved (Yovich *et al.*, 1994). Low ARIC scores may be the consequence of high levels of reactive oxygen species and PF is known to suppress their formation.
(ii) Severe asthenozoospermia. PF has a variable effect on motility as measured by eye under light microscopy. However, CASA studies will often show PF to produce an improvement in the proportion of hyperactivated sperm (Tesarik, Thebault & Testart, 1992) and such cases will usually benefit by its addition in the sperm preparation.
(iii) Previous failed or poor fertilization. There are quite variable causes for this phenomenon and only some will be corrected by PF. It is wise to evaluate such cases carefully and be guided by PF enhancement of the ARIC score or hyperactivation. If one has the facility for measuring reactive oxygen species, this may be another group to benefit. Otherwise improvement should not be expected.
(iv) MESA. PF has proven invaluable in the preparation of epididymal sperm for fertilization (Ord *et al.*, 1992). It has sometimes stimulated motility in 'dormant' spermatozoa and has significantly enhanced the fertilization rate when added either before or after sperm cleanup with mini-Percoll or by

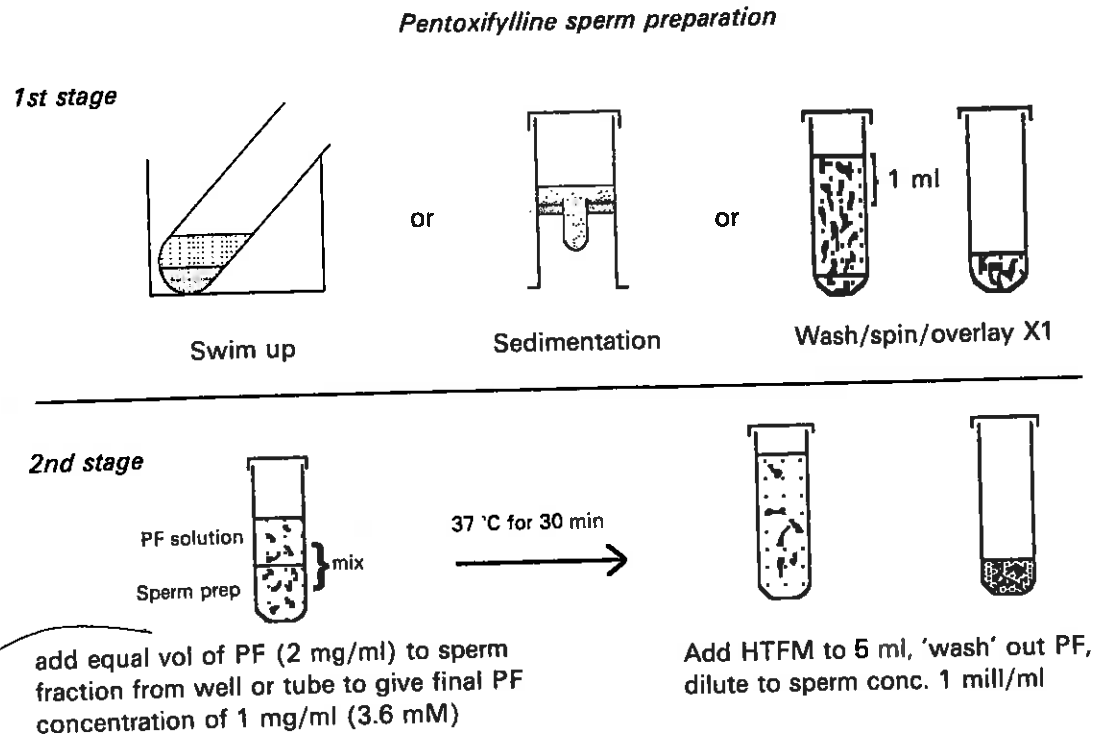

Fig. 6. Pentoxifylline is indicated on the basis of sperm function tests rather than semen analysis parameters. Therefore any appropriate sperm recovery technique is suitable for the first stage. Thereafter the sperm prep is incubated in 3.6 mM PF for just 30 mins prior to washout and immediate insemination. This provides better results than prolonged incubation; however MESA cases may require PF addition before the mini-Percoll stage to 'enliven' dormant sperm in some situations.

sedimentation. The addition of 2-deoxyadenosine often appears to be beneficial in MESA cases.

(v) Micromanipulation. Preparations for partial zona dissection, subzonal insemination and intracytoplasmic sperm injection have aimed to achieve small numbers of hyperactivated sperm in a very clean suspension on a cavity slide or in a culture well. PF, with or without added follicular fluid, can be beneficially used routinely to maximize the chance of inducing the acrosome reaction.

(vi) Caution is advised against the routine use of adding PF to all semen preparations. Normospermic cases will often show a reduced fertilization rate with PF, probably representing a burn-out phenomenon due to the raised intracellular cAMP levels adversely affecting processes which were already proceeding optimally. Some oligo/asthenozoospermics will also show an adverse response hence one should now be guided by the ARIC test and other preliminary studies.

Conclusions

The rationale behind the application of a range of sperm preparation techniques have been presented. These may all be part of daily routines in busy units which can apply flexible management protocols to achieve optimal results. However one must also caution against smaller units introducing this approach as the number of variables which may cause an adverse effect within the overall programme, increases markedly. Trouble-shooting with limited resources under these circumstances can be a tediously frustrating exercise. Many such units will therefore often settle for a standardised approach, e.g. two-layered Percoll sperm preps, aiming for consistent, albeit perhaps slightly suboptimal, results.

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