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Sperm Preparation Methods

Andrology Lab Corner

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The spermatozoa of all placental (eutherian) mammals, including humans, are in a protective, nonlabile state at ejaculation and are incapable of fertilization even if they are placed in direct contact with an oocyte. Consequently, they must undergo a subsequent period of final maturation during which they acquire the capacity to interact with the oocyte–cumulus complex and achieve fertilization. This process, which was discovered independently by Austin and Chang in 1951, was termed *capacitation,* and spermatozoa in the ejaculate are prevented from undergoing capacitation by one or more decapacitation factors that are present in the seminal plasma (Yanagimachi, 1994). Capacitation of eutherian spermatozoa is essential for fertilization not only in vivo but also in vitro, and underlies the manipulation of spermatozoa for clinical in vitro fertilization (IVF).

Not only does seminal plasma contain one or more decapacitation factors that prevent spontaneous capacitation of spermatozoa upon ejaculation, but it also contains one or more factors to which prolonged exposure has adverse effects on sperm function, including the ability to penetrate cervical mucus (Kremer, 1968), undergo the acrosome reaction in vitro, and the fertilization process in general (Rogers et al, 1983; Mortimer and Mortimer, 1992; Mortimer et al, 1998). Consequently, in order for eutherian spermatozoa to have the capacity to fertilize an oocyte, they must be separated from the seminal plasma, and hence, the separation of human spermatozoa from seminal plasma is an essential prerequisite for them to be able to achieve capacitation and express their intrinsic fertilizing ability. In assisted reproductive technology (ART) laboratories, this need is manifested in the process commonly referred to as ''sperm washing,'' in which spermatozoa are somehow removed from the seminal plasma and resuspended in culture medium.

Prolonged exposure (>30 minutes) to seminal plasma after ejaculation can permanently diminish the fertilizing capacity of human spermatozoa in vitro (Rogers et al, 1983), and contamination of prepared sperm populations with only traces of seminal plasma can diminish, or even totally inhibit, their fertilizing capacity (Kanwar et al, 1979). Therefore, spermatozoa for clinical procedures such as intrauterine insemination (IUI) or IVF (and also for laboratory tests of sperm fertilizing ability) must be separated from the seminal plasma environment not only as soon as possible after ejaculation (allowing for the required wait for liquefaction) but also as efficiently as possible.

There are 4 basic approaches to sperm preparation: 1) simple dilution and washing, 2) sperm migration (either directly from liquefied semen from a suspension of washed spermatozoa or from a washed sperm pellet), 3) ''selective'' washing procedures (using density gradients), and 4) adherence methods to eliminate debris and dead spermatozoa (eg, glass wool, glass beads, and Sephadex columns). These have been extensively reviewed elsewhere (Mortimer and Mortimer, 1992; Mortimer, 1994a,b). In the late 1980s, Aitken and Clarkson (1988) discovered that the centrifugal pelleting of unselected human sperm populations often resulted in the generation of free radical or reactive oxygen species (ROS) within the sperm pellet that could adversely affect sperm function in vitro. Moreover, I prepared a detailed review of the literature of in vitro tests of human sperm function and IVF that clearly demonstrated that sperm preparation methods that included such a centrifugation step could impair sperm function to such an extent as to cause a decrease in fertilization rates and even cause fertilization failure in more extreme cases (Mortimer, 1991). The basic conclusion was that the potentially hazardous practice of washing spermatozoa by centrifuging unselected sperm populations should be abandoned in favor of known ''safe'' practices, such as direct swim-up from semen and density gradient centrifugation techniques. Given the association between the term ''sperm washing'' and the simple, usually repeated centrifugation and resuspension of spermatozoa in culture medium, it seems appropriate to urge that the method be reserved for that type of process and that another term, such as *sperm preparation,* be employed when describing processes such as swim-up migration and density gradient centrifugation.

The purpose of this article is to review the significant literature on sperm preparation published since 1990 and update the recommended approaches for human sperm preparation within the context of modern ART practices.

Is Centrifugation Really Harmful?

In simple physical terms, centrifugation can be harmful to human spermatozoa, but this does not seem to be a

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problem until forces in excess of 800 × *g* are applied (Jeulin et al, 1982). After a 1991 editorial (Mortimer, 1991), several laboratories investigated the issue of such ROS-induced damage, and although some researchers approached the question scientifically using hypothesis-testing experiment designs (eg, Chan and Tucker, 1992), others sought to dismiss the idea by illustrating that in their particular situation—usually working primarily with non–male-factor semen samples or with poorly defined ''abnormal semen parameters''—it was not a statistically significant problem (eg, Englert et al, 1992). Another, albeit unrelated, paper by Morales et al (1991), demonstrated that although Percoll gradients exhibited higher sperm recovery, there were no apparent differences in sperm function (zona-free hamster egg penetration, inducible acrosome reactions, and sperm–zona pellucida binding) between such spermatozoa and parallel aliquots prepared by wash-and-swim-up procedures. However, the study was performed on a series of 12 semen samples from normal, fertile men. None of these subsequent studies addressed the real issue by investigating patients with likely iatrogenic sperm dysfunction, and all left findings such as those of Guérin et al (1989) on a large series of IVF patients as incontrovertible evidence of induced sperm dysfunction. Our now greatly increased understanding of the mechanisms by which morphologically abnormal spermatozoa with retained spermatid cytoplasm and leukocytes present within the ejaculate generate free radicals in vitro (see later discussion) provides mechanistic explanations for this problem. It will certainly not affect every man, especially those with (more) normal sperm quality, but in the infertility clinic setting, these men are a minority, and the majority of such patients *must* be considered to be at risk of damage to their spermatozoa during preparation for ART.

Therefore, the earlier conclusion remains valid: centrifugal pelleting of unselected populations of human spermatozoa causes irreversible damage to the spermatozoa that can impair—even totally destroy—their fertilizing ability. Hence techniques that involve any simple sperm washing step in which semen is diluted with culture medium and centrifuged, regardless of whether the pellet is just washed and the spermatozoa resuspended or the motile spermatozoa allowed to swim out from the washed pellet, must be recognized as potentially harmful and sometimes lethal to the spermatozoa being prepared—and consequently, must be abandoned for any application that requires physiological functionality of the prepared spermatozoa. Alternative sperm preparation methods, such as direct swim-up from liquefied semen (with a subsequent centrifugal washing step into fresh medium being safe because the ROS-generating cells have been excluded from the prepared population), some adherence methods, or density gradient centrifugation, must instead be employed. Which of these approaches might be preferable is also discussed later.

Although the success of a sperm preparation method is often assessed by its yield of motile spermatozoa, it is also vital that sperm preparations for clinical use should be free of any microbiological contaminants present in semen. Other relevant considerations in choosing a method include its technical complexity as well as its costs in materials, apparatus, and time. Any possible exposure of spermatozoa during preparation to deleterious influences that may cause iatrogenic sperm dysfunction must obviously be avoided at all costs.

"Safe" Methods for Sperm Preparation

Although the list of techniques described in this article is not exhaustive, the principles used to select them can be applied to determine whether any apparently similar approach is likely or not to be ''safe'' (ie, whether it avoids the risk of ROS generation).

Direct Swim-up From Semen

This is the original swim-up technique that has been used by investigators who study mammalian sperm physiology since at least the 1950s. Liquefied semen is layered beneath a culture medium (or culture medium layered over the semen), and during a subsequent incubation period that can range from 15 to 60 minutes depending on the application, the progressively motile spermatozoa migrate from the semen layer into the culture medium. The inclusion of this migration step is considered to be functionally equivalent to the process by which human spermatozoa escape from the ejaculate and colonize the cervical mucus (Mortimer et al, 1982; Katz et al, 1990; Mortimer, 1995; Mortimer, 1997), although some differences exist because of the different rheological characteristics between the culture medium and midcycle cervical mucus. The selection of spermatozoa for their motility and morphology during in vitro migration is highly comparable (Mortimer et al, 1982), but the process might be suboptimal for clinical applications because of differences in chromatin quality using this method.

The Sperm Select System (Select Medical Systems, Williston, Vt) employs a high-purity preparation of 3000 kd sodium hyaluronate at a 1-mg/mL final concentration in culture medium. In a clinical IVF program, swim-up from semen into the hyaluronate solution gave a significantly higher percentage of motile spermatozoa compared with the traditional swim-up from a washed pellet method and, ultimately, allowed the achievement of a higher pregnancy rate (Wikland et al, 1987). Whether these improved results were due specifically to the use of the hyaluronate or to the use of a method that did not involve the initial

centrifugal pelletting was not ascertained. More recent research has demonstrated numerous beneficial effects of hyaluronate upon spermatozoa (Huszar et al, 1990; Sbracia et al, 1997), and therefore Sperm Select, especially in view of its market focus toward office gynecologists who perform IUI, must be considered a useful technique for clinical human sperm preparation (Zimmerman et al, 1994). However, it is important to undertake additional studies of spermatozoa prepared in this way to assess their deoxyribonucleic acid (DNA) stability and chromatin damage.

Adherence Methods

These methods should not be directly deleterious to spermatozoa as long as they do not include a prewash step, although methods that employ glass fibers (fragments of which can contaminate the final product) should be carefully considered before they are accepted for clinical applications such as preparing spermatozoa for IUI. Only limited studies of sperm selection and the functional competence of the prepared populations are available that compare adherence methods with other techniques such as direct swim-up from semen (DSUS) and density gradients, although the second generation of SpermPrep columns (SpermPrep, ZBL, Lexington, Ky), which do not require that the semen be prewashed, have been reported to provide poorer yields (Smith et al, 1995).

Density Gradients

Early studies (Beatty, 1964) reported that although colloidal silica allowed isopycnic separation of spermatozoa, the fertility of these cells exhibited problems, and it was only with the advent of modified colloidal silica in the late 1970s that this method became useful. Coating silica particles with polyvinylpyrrolidone (PVP) was introduced by Håkan Pertoft (Pertoft et al, 1978) and commercialized as Percoll (Pertoft's Colloid), which was used to make density gradients for sperm preparations in the early 1980s. This was a major innovation that to a large extent dominated clinical and experimental human sperm preparation until Percoll was withdrawn from clinical use by its manufacturer (Pharmacia Biotech, Uppsala, Sweden) in 1996 (additional information on this appears later). Several intrinsic properties of colloidal silica made Percoll (and its recent replacements, PureSperm and ISolate) ideal for preparing density gradients for selecting human spermatozoa. First, as a mineral substance, it has no osmotic effect when added to culture medium; second, it allows high-density (high specific gravity) media to be prepared, which is important because normal, mature eutherian spermatozoa are dense cells; and third, being a colloid rather than a solution, it has low viscosity and thus does not retard sperm cell sedimentation. Although some authors reported the use of continuous Percoll gradients in the early days (eg, Gorus and Pipeleers, 1981; Bolton and Braude, 1984), clinical applications since the late 1980s have almost exclusively employed discontinuous gradients (eg, Arcidiacono et al, 1983; Lessley and Garner, 1983; Dravland and Mortimer, 1985). Discontinuous gradients are usually prepared with 2 or 3 layers, although methods that use up to 12 layers have been reported for special applications. Other classical density gradient materials, such as sucrose, cesium chloride, Ficoll, and Metrizamide, failed because dense solutions were hypertonic and highly viscous.

Nycodenz, which is based on the iodinated cyclic hydrocarbon iohexol, was found to be useful in making density gradients suitable for human sperm preparation (Gellert-Mortimer et al, 1988) and is the basis of OptiPrep (Nycomed Pharma, Oslo, Norway). A dimeric form, iodixanol, is also used to make density gradients for eutherian, including human, spermatozoa (Accudenz, Nycomed Pharma), which appear to perform quite well (Sbracia et al, 1996; Smith et al, 1997), although its osmotic activity requires that the medium in which it is prepared have a different ionic composition to the usual media used for sperm preparation and is known to support capacitation and fertilization in vitro.

The plant-derived molecule arabinogalactan has also been used to prepare density gradients for human spermatozoa under the trade name IsoCare (InVitroCare, San Diego, Calif), although this author is not aware of any published studies that compare the resulting sperm populations with other techniques for sperm function or clinical IUI or IVF.

Although Percoll was based upon colloidal silica coated with PVP, the replacements, PureSperm (Nidacon International AB, Göteborg, Sweden) and ISolate (Irvine Scientific, Santa Ana, Calif) use colloidal silica with covalently bound silane molecules (silanized silica). Consequently, the characteristics of these colloids should be at least the same as for equivalent Percoll preparations (Perez et al, 1997), and their clinical utility seems to be at least as good, if not better, than Percoll (Chen and Bongso, 1999). The major difference is that whereas Percoll was sold as colloidal silica in a weak inorganic buffer solution (and was typically used in conjunction with a 10× buffer to make ''isotonic'' 90% vol/vol Percoll), PureSperm and ISolate are prepared in what is effectively an isotonic, ready-to-use culture medium. There are now many other products based upon silanized silica on the market, and these are likely to be third-party products based upon similar raw materials that are used to make PureSperm and ISolate. An example of these products is the Enhance cell isolation product from Conception Technologies (San Diego, Calif).

The Percoll Saga

In October 1996, many laboratories worldwide received a letter from Pharmacia Biotech that declared that Percoll was not to be used for clinical applications and that its use for these purposes was to be prohibited, effective January 1, 1997. The letter from the manufacturer stated, ''Pharmacia Biotech recognises the ethical and legal obligations which compel us to restrict the use of Percoll to RESEARCH PURPOSES ONLY and to take measures to ensure it is not used for the isolation of cells which will be subsequently used for clinical purposes in humans.'' This indicated that the manufacturer was not specifically targeting ART laboratories, but the warning was meant for anyone who was isolating cells that would be used for clinical purposes for humans, including stem cell therapy.

This caused quite a furor among Internet special interest groups, such as Androlog (androlog@godot.urol.uic.edu), ARTlog (artlog@kumc.edu), and EmbryoMail (EmbryoMail@lpsi.barc.usda.gov). Many researchers asked why the step had been taken; in particular, many believed that because they had used Percoll ''safely'' for so many years, they could continue to use it regardless of any pronouncement by Pharmacia Biotech, especially if they bought their Percoll supply from another company, such as Sigma Chemical Company (St Louis, Mo). However, because Pharmacia Biotech is the sole manufacturer of Percoll, companies such as Sigma were buying it in bulk and repackaging it. There was also concern that many companies were making ready-to-use gradient kits based on Percoll; yet such kits were all marked ''For In-Vitro Use Only'' or ''For Research Use Only,'' and none were sold for the specific application of sperm preparations for use in human clinical ART. Furthermore, it was widely recognized that some batches of Percoll contained high levels of endotoxin contamination, substantially higher than those permissible for in vivo administration, which could adversely affect sperm survival or development of fertilized oocytes. The situation was particularly obscure in the United States because at that time, the U.S. Government did not recognize IVF. At the same time, although the U.S. Food and Drug Administration (FDA) licensed products for use in IUI, it did not license products that mentioned IVF and, hence, these descriptions were generally seen as a labeling tactic. Many workers claimed that Pharmacia Biotech did not have the right to stop them from using Percoll in this way; obviously, this assertion was misguided and incorrect, and anyone who continued to use Percoll after the prohibition date ran commercial and medico-legal risks.

The July 1997 issue of *The Embryologist*, the newsletter of the British Association of Clinical Embryologists (ACE), contained an article by David Morroll, a member of the ACE Executive Committee, who reviewed an independent legal opinion on the need to use ART-specific products. In his article, Morroll stated that, ''if a manufacturer states that its products may be used *only* in one way (e.g., for research purposes only), deviations from this are entirely at the risk of the user,'' and Morroll concluded, ''should one choose to use such a product in an unauthorised manner and a problem arose due to its use, any defence would be non-existent.'' Morroll also stated, ''It may even be argued that one is guilty of a breach of duty of care. In addition, the manufacturer may opt to take legal action if its product is used in an unauthorised way. . . .if a non-ART product is used and is linked with a death, this could in theory lead to criminal proceedings, probably on the grounds of unlawful killing but possibly manslaughter if considered reckless.'' Hence, the legal opinion in the United Kingdom even 3 years ago recognized that the onus was on ART clinics and researchers to use ART-specific products exclusively, although an ACE survey at the time revealed that only 2 clinics in the United Kingdom had implemented such a policy, even though several had indicated that they were considering changing their current policy.

In 1996, Sbracia et al expressed concern that Percoll was not approved by FDA and that the manufacturer had not intended the product to be marketed for sperm preparation. Near this time, FDA was preparing to regulate all ART products, and assurances of raw-product purity and suitability would have been required for an FDA 510(k) submission. As the debate continued, I ventured an opinion on EmbryoMail 732 (April 2, 1998) that Pharmacia Biotech's prohibition could have come about because third-party companies that assembled Percoll kits were seeking assurances that the raw materials in their preparations were pure and suitable. This might have rung alarm bells at Pharmacia Biotech. Certainly this was a likely scenario, and because Percoll was manufactured primarily as a research-grade product and the ART market represented only a tiny proportion of the total Percoll market, there would have been no sound commercial grounds for Pharmacia Biotech to upgrade its manufacturing processes to those that would have been essential to supply a medical product. In addition to these technical and practical issues, the cost of Percoll would have skyrocketed for non-ART users and possibly destroyed that segment of Pharmica Biotech's market.

I have heard references to ''a report from Europe'' that PVP might cause genetic abnormalities in embryos, and whereas this has been associated with the use of PVP to immobilize spermatozoa during an intracytoplasmic sperm injection (ICSI) procedure, its use in coating the silica particles in Percoll (in addition to the free PVP that Percoll was known to contain) could have caused Pharmacia Biotech, FDA, or both to raise concerns regarding any use of Percoll for ART.

The study at the root of this ''report'' is unknown, but it may have been the paper by Ray and colleagues (1995) that included 2 confused references, 1 to an earlier paper by Fishel and colleagues (1993) on the potential dangers of microassisted fertilization and another to an abstract by Bras and colleagues (1994), which said that ''some'' PVP solutions (from anonymous suppliers) were toxic and caused failed embryonic development after injection into mouse oocytes. However, the paper by Fishel and colleagues made no mention of PVP, and besides, any association between a particular commercial PVP product and abnormal embryo development is an entirely separate issue! Moreover, the study by Ray and colleagues found no evidence that PVP or methyl cellulose caused DNA lesions and concluded that their data provided reassuring evidence for the use of those products in sperm injection procedures.

A third consideration is Percoll's variable and sometimes high endotoxin levels. This can be tested for by end users, and only ''safe'' batches can be used in clinical procedures. However, this would continue to make Percoll an unreliable raw material according to good manufacturing practices, standards by which all medical device manufacturers must operate, and would elevate a clinic's costs by purchasing unusable batches of the material.

Percoll has been used, apparently safely, for many years in many ART laboratories (although it will remain a mystery as to whether any poor or failed fertilization cases or poor or failed embryonic development might have been caused by undetected, elevated endotoxin levels in Percoll). But the next-generation products that are based on silanized colloidal silica particles must be manufactured according to good manufacturing practices for clinical use, for which Percoll was never intended. In addition, new products should receive regulatory approval from the FDA for at least IUI use, and preferably unrestricted ART, as well as clearance for use as a medical device by other regulatory authorities, including the European Medical Device Directive. The attendant increased cost of such products and the task of obtaining international regulatory approvals is attributable to the need to follow these higher manufacturing standards, but the cost should be only about $5 per procedure (presuming a fair price is set by distributors), which is an insignificant cost within the IUI cycle and absolutely trivial in view of the cost of IVF. Regardless of budget squeezes on ostensibly commercial grounds, everyone involved in medical care must strive for best practice, and the replacement of Percoll by products such as PureSperm or ISolate has to be viewed in this light. And those laboratories that still use Percoll must question their motives and balance the small savings they are achieving against the risks of using an unregistered product whose manufacturer has declared it to be unsuitable for ART applications. The potential liability issues must be huge, and surely no one wants to be part of a possible criminal litigation.

Medium or Buffer?

Because the centrifugation step is performed in air rather than in a CO_2-enriched atmosphere, it is recommended that density gradients (and swim-ups) use a HEPES-buffered medium (often referred to as a sperm wash buffer) rather than a bicarbonate-based medium (a sperm wash medium). However, because sperm capacitation requires the presence of significant concentrations of bicarbonate ions, the washing step and final resuspension must be made into a medium; otherwise, in vitro capacitation and hence fertilization can be compromised. For IUI preparations, a buffer can be employed because it will be greatly diluted after insemination, and because if the spermatozoa undergo capacitation in vitro during prolonged incubation before insemination, their hyperactivated motility could compromise their ability to traverse the uterotubal junction (Shalgi et al, 1992; Mortimer, 1997), but if insemination is to be performed soon after sperm preparation, then a medium can be safely used.

Should We Include Antioxidants in Sperm Preparation Media?

Because spermatozoa may be exposed to potentially hazardous effects of ROS during preparation, several workers have suggested including antioxidant protection (eg, glutathione) in sperm preparation media formulations. Although a slightly improved yield supports this in preliminary evidence (Parinaud et al, 1997), more extensive studies will be needed before the value of this concept can be established.

Assessing the Yield of a Sperm Preparation Method

The success or applicability of a sperm-washing method can be considered in terms of either the absolute or relative yield of motile spermatozoa that one obtains at the end of the technique. Usually, progressive motility is used for this purpose because nonprogressive spermatozoa are unlikely to be potentially functional (except perhaps for ICSI).

Relative yield is the proportion of progressively motile spermatozoa submitted to a preparative procedure that are present in the final preparation. It is calculated the following way:

$$\text{yield (\%)} = (v \times c \times \text{pm\%})/(V \times C \times \text{PM\%}) \times 100,$$

where v is the final preparation volume, V is the volume of semen used, c is the sperm concentration in the final preparation, pm% is the prepared sperm population progressive motility, C is the semen sperm concentration, and

PM% is the progressive motility of spermatozoa in the semen.

Absolute yield is the total number of progressively motile spermatozoa that can be obtained if the whole ejaculate is used, although an allowance is usually made for the aliquots needed to perform a standard semen analysis (eg, 0.3 mL). Yield quality is, however, of vital importance, especially if the product is to be used to create embryos for clinical purposes.

Modifying the Yield by Altering the Colloid Concentration

This section considers how the colloid concentration and osmolarity of the gradient influence yield. Because much of this research was performed several years ago, it employed Percoll and has been presented on this basis. This must not be taken as any suggestion that Percoll should be used for any clinical application nowadays. In simple terms, the purchased Percoll product can be taken as 100% colloid and equivalent to, for example, 100% PureSperm for any application of these principles.

The earliest Percoll method we used had a true 95% vol/vol concentration of Percoll in its bottom layer (Dravland and Mortimer, 1985), and although it provided excellent yields when used with research donors, in clinical specimens from an infertility clinic, the yield was much lower, sometimes approaching zero. Empirical studies demonstrated that optimum clinical yields could be obtained with about 80% vol/vol in the lower Percoll layer, and whereas 72% would often result in at least slight increases in the absolute yield, it seemed to be at the expense of recovering more abnormal spermatozoa (Mortimer, 1994b). Consequently, we always recommend the use of a lower layer of 81% Percoll to obtain optimum yields (obtained as 90% of the 90% isotonic Percoll). This procedure was essential when working with Percoll because the osmolarity of true 100% (ie, the stock product as supplied in the bottle from Pharmacia or Sigma is about 17 mOsm, so it was mixed as 9 + 1 with a 10× medium so that if the medium was, say, based upon Quinn's HTF at 285 mOsm, the isotonic Percoll would have an osmolarity of 258 mOsm; that is, ([9 × 285] + 17)/10. However, the incorrect practice by some authors of renaming the 90% isotonic Percoll preparation as a 100% preparation has created enormous confusion. These osmotic shifts were the generally accepted explanation of why spermatozoa recovered from a Percoll gradient and then washed in fresh medium (at 285 mOsm) seemed to swim with jerky movements for the first few minutes after final resuspension while they were recovering from the combined osmotic shock of going from about 340 mOsm in the seminal plasma to 258 mOsm in the lower gradient layer and back to 285 mOsm in the culture medium, in addition to the general effect of centrifugation.

Several laboratories reported the use of hypertonic Percoll gradients that were designed to minimize such osmotic shocks (Velez de la Calle, 1991; Mortimer, 1994b), and whereas postrecovery sperm behavior was more normal, the yield was more variable. This was because of the basis upon which density gradients operate and the highly variable morphology of spermatozoa in the ejaculates of different men. Because density gradients operate on the basis of cells' specific gravity, with centrifugation causing them to move down the gradient to their isopycnic point, the presence of variable amounts of retained cytoplasm in the spermatozoa will cause them to have different densities. Normal spermatozoa with no retained cytoplasm are very dense because of their condensed chromatin, and they reach the bottom of the centrifuge tube because their density is slightly greater than that of the lower or lowest layer (about 80% Percoll in this discussion). Therefore, because research donors are selected on the basis of their excellent semen characteristics, they should have the highest proportions of very dense spermatozoa and will provide good yields, even with a 95% lower layer—and semen specimens from patients who are infertile are likely to perform substantially less well. Reducing the lower layer to 72% Percoll allows less dense spermatozoa to reach the bottom of the tube, which results in an increase in total yield, but only because spermatozoa with some retained cytoplasm are recovered.

For the aforementioned reasons and because the modern products such as PureSperm and ISolate are sold as isotonic colloidal preparations that eliminate the need for the 10× mixing step, all workers are urged to consider how they report the composition of their gradients, to use scientifically correct descriptions, and thus to avoid the confusion that can result in the use of inappropriate gradient formulations (eg, the protocol for a Percoll-based gradient published in the third edition of the WHO laboratory manual [World Health Organization, 1992] that had a 72% lower layer; that is, 80% of 90%).

Another important point is that cryopreserved spermatozoa are in a highly hypertonic medium and hence will suffer extreme osmotic shock upon entering the upper layer of a density gradient. This is the explanation for the obligate requirement of a slow dilution of cryopreserved semen after thawing with a large volume of culture medium so as to bring the osmolarity of the sperm suspension closer to that of the gradient before beginning the first centrifugation step (Ford et al, 1992).

Modifying the Yield by Altering the Centrifugation Speed

All early reports of Percoll gradients employed an initial centrifugation step through Percoll at 300 × *g*, which empirically had been found to provide optimum sperm recovery (Dravland and Mortimer, 1985). Increasing the centrifugation speed, time, or both could sometimes in-

crease the number of spermatozoa recovered, but not necessarily substantially—or usefully, in terms of the quality of the extra spermatozoa recovered. Over many years of experience we have found there to be no real benefit in altering this initial centrifugation step from 20 minutes at 300 × *g*, and we continue to make this recommendation. Because the purchased Percoll product (100% colloid) is equivalent to 100% PureSperm, the same optimized performance can be expected using the same centrifugation conditions with this product, and because ISolate is described as being the same as 100% Percoll, one would expect the same for that product as well (although I have never used ISolate).

The subsequent wash centrifugation step should be sufficient to recover the great majority of the previously pelleted spermatozoa without exposing them to excessive centrifugal force. In practice, this has been achieved at 500 × *g* for 10 minutes; again, longer centrifugation does not provide any significant benefit, although a slightly shorter time of, say, 6 minutes can be used if the speed is increased to 600 × *g*. Lower speeds can be used, but there is an increased risk of losing some of the spermatozoa in the discarded supernatant, and whether there are fewer good spermatozoa with lower densities is unknown. Under no circumstances should the speed exceed 800 × *g*.

Reports must not describe their methods using revolutions per minute because they are highly influenced by rotational radius and the centrifugal forces discussed in the previous paragraph are actually g_{max} values that were calculated for what the spermatozoa would experience at the bottom of the centrifuge tube. The formula describing the relationship between rotation speed, rotational radius, and centrifugal force is as follows:

$$g = 0.0000112 \times r \times N^2, \text{ or } N = \sqrt{(g/[0.0000112 \times r])},$$

where g = the maximum centrifugal force achieved at the bottom of the tube, r = the rotational radius (in centimeters), and N = revolutions per minute.

Do Spermatozoa Swim Through the Gradients?

Several workers have commented that spermatozoa swim through the density gradients and that the centrifugal force helps align them in a downward direction, a concept that may be based on some reports of using simple migration through density gradients under unit gravity. Additional centrifugal force will certainly cause human spermatozoa to align in a head-down orientation (rabbit spermatozoa do this under unit gravity because of the larger size of their heads; Branham, 1969; Mortimer, 1979), but the relative contributions of sperm motility and settling under a greatly increased gravitational force are clearly unequal—and furthermore, the less dense spermatozoa never reach the bottom of the gradient but remain at their isopycnic level. So the conclusion, on the basis of simple physics, is that the separation of spermatozoa on density gradients based on colloidal silica are true isopycnic separation methods but are perhaps unusual in that they cannot achieve sufficiently high densities (at least using currently available colloidal silica materials) to separate the spermatozoa as a discrete band above the bottom of the tube.

Sperm Selection

Sperm Motility and Morphology

Any method based upon sperm migration will certainly produce a sperm population that is selected for improved sperm morphology. However, this has long been known to be based upon the differential distribution of midpiece and tail defects among spermatozoa with normal and abnormal head morphology (Mortimer et al, 1982), so that applying a selection pressure based upon motility will create a concomitant improvement in overall sperm morphology. This process is equivalent to that occurring at the level of penetration of the cervical mucus in vivo and reflects a natural process of sperm selection. However, it has also long been established that this process of sperm phenotypic selection does not provide a general selection for spermatozoa with normal genotype, except perhaps for reductions in diploid spermatozoa that have larger heads (Carothers and Beatty, 1975).

Sperm Morphology (Phenotype) and Genotype

There are few instances in which sperm morphology assessed by light microscopy is related to the genetic content of the spermatozoa. Abnormally small and large sperm heads are often associated with aneuploidy and perhaps diploidy (Carothers and Beatty, 1975) and the *t* loci in mice cause the production of abnormal spermatozoa (Olds-Clarke, 1990). More specific associations also exist; for example, the total lack of sperm motility in men with Kartagener (or immotile cilia) syndrome and the defect known as globozoospermia, although their patterns of inheritance remain unknown, notably because hitherto, men with these defects were sterile. Reports of morphometric differences between X- and Y-bearing spermatozoa—and futile attempts to separate the 2 populations of spermatozoa based upon them (Gledhill, 1988; Martin, 1994)—are numerous, and whereas specific size differences seem to exist (Cui, 1997), they are too small to have practical consequence (although they do confirm the association between sperm head size and total chromosome content).

Several authors have expressed concern over the use of ICSI, in which spermatozoa do not go through the same selection process as they would in vivo and hence may contribute to an increased prevalence of genetic anomalies in the resulting offspring. The grounds for this sup-

posed relationship are, however, generally baseless at the light microscopy level. It has been shown that morphologically abnormal spermatozoa carry normal karyotypes (Martin and Rademaker, 1988) and can produce normal offspring (Burruel et al, 1996), although it has also recently been reported that human and mouse spermatozoa with abnormal head shape can have an increased incidence of structural chromosomal aberrations (Lee et al, 1996; Kishikawa et al, 1999). Nevertheless, we know that under normal in vivo conception conditions in fertile couples, many genetically abnormal embryos are produced and transmitted via the spermatozoa, many of which are caused by aneuploidy and even triploidy. Consequently, such calls for abandoning ICSI because it does not properly select spermatozoa are alarmist and, on the basis of our current knowledge, largely unfounded.

This subject is a major area of current research and space precludes a more detailed review in this article. Interested readers will find additional discussions in the proceedings of the second Collioure conference *Genetics of Human Male Fertility* (Barratt et al, 1997) and elsewhere (eg, Mortimer, 2000).

Free Radicals and Sperm Chromatin Damage

The deleterious effect of free radicals or ROS upon sperm function and their role in the etiology of male infertility was originally described in John Aitken's laboratory in Edinburgh (Aitken and Clarkson, 1987). Soon afterward, the significance of ROS generation during sperm preparation in vitro was described (Aitken and Clarkson, 1988) and the existence of widespread evidence for the detrimental influence that this could have upon sperm function tests and IVF was collated (Mortimer, 1991). ROS are generated both by leukocytes present in semen and spermatozoa (Krausz et al, 1992; Aitken, 1995; Whittington and Ford, 1999). However, only those spermatozoa with excess retained spermatid cytoplasm generate ROS (Aitken et al, 1994; Aitken, 1995; Huszar et al, 1998, 1999), which illustrates the highly beneficial selection of spermatozoa that density gradients confer by eliminating these less dense spermatozoa from the final preparation. ROS affect not only the sperm plasma membrane by causing phospholipid peroxidation, and hence decreased membrane fluidity and impaired sperm function, but also the sperm DNA by causing strand breaks that can be revealed by various tests of sperm DNA integrity such as nick translation (Sakkas et al, 1997) and the sperm chromatin structure assay (SCSA; Evenson, 1999; Evenson et al, 1999).

Of particular importance in the practice of ART is a study comparing the incidence of DNA nicks in spermatozoa recovered by simple washing, swim-up from semen, and colloidal silica–based density gradient separation (Percoll and PureSperm) in which only the density gradient methods permitted selected populations of spermatozoa with a lower incidence of DNA nicks to be recovered (Sakkas, in press). Other studies using SCSA have shown that swim-up (Spanò et al, 1999) and glass wool filtration (Larson et al, 1999) can select spermatozoa with better chromatin stability. However, because SCSA assesses the susceptibility of sperm DNA to in vitro acid- or temperature-induced damage rather than the actual existence of nicks in the sperm DNA, the relative value of the 2 assays, and hence their clinical significance, remain to be determined.

The vital importance of these observations is that, whereas many ART laboratories employ density gradient preparation methods for their IVF spermatozoa, many use simple washing for ICSI because the risk of ROS-induced sperm dysfunction arising from simple centrifugal washing is considered unimportant because sperm function is generally accepted as irrelevant to fertilization by ICSI. However, the Brussels group that originally developed the ICSI technique employ density gradients (originally Percoll, now PureSperm; see Verheyen et al, 1999). But the sequel to this is that the men who are considered to need ICSI will be more likely to have increased proportions of abnormal spermatozoa, and hence they will also be the most susceptible to ROS-induced sperm DNA damage. Because it has been well established that ROS can cause substantial degradation of the sperm DNA that does not necessarily affect their fertilizing ability (Aitken et al, 1998), spermatozoa prepared by simple washing will definitely be at a much greater risk of contributing a defective genome to the embryo and could underlie the increased developmental failure of ICSI-derived embryos after the 8-cell stage when the embryonic genome is activated (Shoukir et al, 1998).

Conclusion

According to our current knowledge, colloidal silica density gradients must be considered the most appropriate and generally applicable clinical sperm preparation technique. If other methods, such as some of the adherence-based products, are able to separate spermatozoa with comparably reduced levels of DNA damage, then this must be demonstrated through independent scientific study. Indeed, it would seem essential that any future studies on the development of new sperm preparation methods or that report comparative analyses of various methods must include sperm DNA assessments in order to have real clinical value. Moreover, because the most fundamental guiding principle of medical care is *primum non nocere*, or first, do no harm, physicians and clinical scientists who participate in the management of ART programs are obligated to avoid techniques that have known hazards if other, safer techniques are available. It seems

highly unlikely that any couple undergoing ART would choose to save $5 by using a simple sperm-washing technique instead of density gradients if the true risks, in terms of an increased chance of embryonic wastage resulting from an elevated incidence of genetic anomalies in the embryos, were fully explained during the consent process. It is clearly important that clinical units use only sperm preparation products that are designed and approved by relevant (ie, local) regulatory authorities for ART use.

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