Application of modern molecular techniques to evaluate sperm sex selection methods

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The aim of this paper is to review modern approaches which have been used to evaluate sex pre-selection procedures. Two approaches can be used, polymerase chain reaction (PCR) and fluorescence in-situ hybridization (FISH). FISH is currently the method of choice for evaluating sex selection procedures because: (i) FISH accurately identifies the sex chromosome of individual spermatozoa using specific probes for the X and Y chromosomes and a two-colour detection system; and (ii) large numbers of spermatozoa can be screened in a short period of time. Of the published sex pre-selection methods tested using FISH, only flow cytometry has been shown to produce a clinically significant enrichment of X- and/or Y-bearing human spermatozoa. Studies have shown that 12-step Percoll gradients produce a slight but clinically insignificant enrichment of X-bearing spermatozoa, swim-up techniques do not appear to enrich either X- or Y-bearing spermatozoa, and discontinuous albumin gradients do not enrich Y-bearing spermatozoa. Despite this evidence, some of these methods continue to be used clinically, so it is vital that sex selection methods are properly evaluated using reliable methods such as double-label FISH before they are introduced for clinical use.

Key words: FISH/PCR/sex selection/X chromosome/Y chromosome

Introduction

During meiosis in the testis, the sex chromosomes segregate into individual spermatocytes, and haploid spermatozoa therefore carry either the X or Y chromosome. There is a 1:1 ratio of X- to Y-bearing spermatozoa in semen and fertilization of an X-bearing haploid oocyte by either an X- or Y-bearing spermatozoon determines the sex of the resulting embryo.

Pre-conceptual sex selection has generated great interest and controversy, especially since the early 1970s when Ericsson et al. (1973) reported that Y-bearing human spermatozoa were enriched by passage through discontinuous albumin gradients. Since then, a modified swim-up procedure was reported to enrich Y-bearing spermatozoa (Check and Katsoff, 1993), Sephadex columns and 12-step Percoll gradients were proposed to enrich X-bearing spermatozoa (Steeno et al., 1975; Iizuka et al., 1987), and flow cytometry has been used to enrich both X- and Y-bearing spermatozoa (Johnson et al., 1993). However, there have been numerous conflicting reports and many investigators have been unable to verify enrichment of X- or Y-bearing spermatozoa using some of these techniques. Furthermore, with the exception of Beernink et al. (1993), clinical trials of these sperm separation procedures have all involved small numbers of conceptions (Dmowski et al., 1979; Corson et al., 1984; Iizuka et al., 1987; Check and Katsoff, 1993) and there has been only one randomized, controlled clinical trial to date which showed no increase in the incidence of male births following the use of the albumin gradient technique (Jaffe et al., 1991). After reviewing the literature on sperm sex selection, Gledhill and Edwards (1993) concluded that ‘Many (methods) are promoted highly by the originators, but none has been confirmed independently or gained wide acceptance; most give inconsistent results’.

In this review, we will describe modern techniques which are used to identify X- and Y-bearing spermatozoa, and discuss studies which have used these techniques to evaluate sperm preparation methods used for sex pre-selection.

Differences between X- and Y-bearing spermatozoa

Currently, sperm sex selection methods all presuppose the existence of fundamental differences between X- and Y-bearing spermatozoa which can be exploited to enrich one population or the other. These differences include the size, density, motility and surface properties of X- and Y-bearing spermatozoa (Gledhill, 1988; Gledhill and Edwards, 1993).

It has been experimentally proven that there is a difference in the DNA content of X- and Y-bearing spermatozoa due to their differential chromosome constitution (Johnson, 1994). A theoretical argument for a difference in the size of X- and Y-bearing spermatozoa was advanced by Roberts (1972), although for many years no repeatable experimental evidence was presented to substantiate the theory (Gledhill, 1988). Recently, Cui and Matthews (1993) addressed this issue using the polymerase chain reaction (PCR) technique. Primers from the putative human testis-determining gene (SRY) on the Y chromosome were used to discriminate human male DNA. Control primers from the human sperm receptor gene (ZP3) on chromosome 7 were used. The SRY primers correctly
identified 41 male and female blood samples and discriminated the sex of 20 individual lymphocytes (from male and female blood) with 100% accuracy. The primers were subsequently tested on human spermatozoa. A total of 895 spermatozoa from eight semen donors were individually collected by micromanipulation and then subjected to PCR amplification using the SRY and ZP3 primers. The amplification rate was 97.3% and a 1:1 ratio of X- to Y-bearing spermatozoa was found. Once the methodology had been validated, photographs of 233 individual spermatozoa were taken and each spermatozoon was then sexed by PCR. The photographs were magnified (×30) by projection and direct measurements of the sperm head and tail were made. Statistical analyses showed that the length, perimeter and area of the sperm head, and the length of the neck, region and tail, were greater in X- than in Y-bearing spermatozoa (Cui and Matthews, 1993). This study demonstrated for the first time that X-bearing spermatozoa are statistically larger and longer than Y-bearing spermatozoa, although there was considerable overlap between the two sperm populations.

One of the assumptions that has been used to justify enrichment of X- and Y-bearing spermatozoa using different preparation procedures is that Y-bearing spermatozoa swim faster than X-bearing spermatozoa and have a greater ability to penetrate viscous solutions and the interfaces between viscous solutions. This hypothesis was invoked by Ericsson et al. (1973) to explain enrichment of spermatozoa with Y bodies (putative Y-bearing spermatozoa) after passage of spermatozoa through albumin gradients. While a difference in the motility of the two sperm populations is often assumed, there is in fact no direct experimental evidence to support this contention.

### Identification of X- and Y-bearing spermatozoa

One of the principal problems in the evaluation of sex selection techniques has been that, until recently, specific and accurate methods for identifying X- and Y-bearing spermatozoa have not been available. The degree of enrichment of X- or Y-bearing spermatozoa after the application of sperm separation techniques has frequently been carried out using quinacrine staining, in which the putative Y-bearing spermatozoa exhibit a fluorescent spot or Y body, and the putative X-bearing spermatozoa remain unstained (Barlow and Vosa, 1970). However, quinacrine produces false positive and false negative results in interphase cells (Thomsen and Niebuhr, 1986) and several studies have shown that this technique can produce misleading and inaccurate results with human spermatozoa. For instance, the incidence of spermatozoa with two Y bodies did not correlate with karyotypic data indicating that some Y bodies in spermatozoa were false positives (Wyrobek et al., 1984). Beckett et al. (1989) found that the incidence of Y-bearing spermatozoa estimated using quinacrine did not accurately reflect the content of Y chromosomes determined using a DNA probe. Furthermore, van Kooij and van Oost (1992) reported that spermatozoa separated on 12-step Percoll gradients were recorded as 86% X-bearing by quinacrine staining but only 50% X-bearing using a DNA probe. These studies demonstrated that quinacrine staining is not a reliable method for determining the gender of human spermatozoa, and in view of the introduction of specific and reliable molecular biology techniques, its use can no longer be justified.

Human spermatozoa can be karyotyped after fusion with zona-free hamster oocytes and this method has also been used to determine the gender of human spermatozoa (Brandriff et al., 1986; Martin et al., 1987; Ueda and Yanagimachi, 1987). While this technique produces excellent karyotypic information (Martin et al., 1987), it is too time-consuming and expensive to use for screening large numbers of spermatozoa to determine their gender. In addition, the spermatozoa that are karyotyped may not be representative of the overall population of spermatozoa because only a small percentage of human spermatozoa fuse with hamster oocytes, and only that sub-set of spermatozoa would be analysed.

With the advent of PCR and in-situ hybridization (ISH), it has now become possible to accurately identify the gender of each individual human spermatozoon, and this has opened the way for truly objective assessment of sperm separation procedures. PCR has been used to determine the ratio of X- to Y-bearing spermatozoa in human semen samples, in sperm populations separated by swim-up or using Sephadex columns (Lobel et al., 1993), and in individual human spermatozoa (Cui and Matthews, 1993). However, while PCR is very specific and highly sensitive, its application to populations of cells is of limited use in the evaluation of sex selection procedures, and it is too labour intensive to be used for screening large numbers of individual spermatozoa isolated by such procedures.

ISH is a technique in which chromosome-specific DNA probes are hybridized to chromosomes in cells and are then visualized using reagents which produce a coloured, fluorescent or radioactive signal. ISH can be used on cells at either interphase or metaphase, so it is well suited to the detection of chromosomes in interphase sperm nuclei. Early studies used autoradiographic detection systems (Joseph et al., 1984), but more recently, fluorescence in-situ hybridization (FISH) has become the method of choice for studying chromosomes in spermatozoa. FISH has a number of advantages over techniques such as PCR and quinacrine staining. Firstly, chromosome-specific DNA probes are used so there is no question about the validity of the signals. Secondly, individual spermatozoa are analysed rather than populations of spermatozoa. Thirdly, large numbers of spermatozoa can be scored quickly and accurately. Finally, aneuploid spermatozoa (XX, YY, XY) can be detected if two or three different probes are simultaneously hybridized to the cells.

FISH using a single probe to either the X or Y chromosome has been used to confirm that the ratio of X- to Y-bearing spermatozoa in human semen is 1:1 (Guttenbach and Schmid, 1990; Wyrobek et al., 1990; Han et al., 1992). Single label FISH has also been used to evaluate human sperm fractions recovered from albumin gradients and Sephadex columns (Vidal et al., 1993). However, when only a single probe is used, only some of the spermatozoa exhibit a hybridization signal and an assumption has to be made that the unlabelled spermatozoa carry the other sex chromosome. This is an
unacceptable assumption because spermatozoa can remain unlabelled for several reasons: (i) they carry the other chromosome; (ii) they are aneuploid and do not carry either sex chromosome; or (iii) hybridization failed to occur. Furthermore, the overall hybridization efficiency cannot be determined using single label FISH, so the proportion of spermatozoa that were unlabelled due to hybridization failure cannot be determined. To obtain unequivocal identification of X- and Y-bearing sperm, it is necessary to use a double label FISH procedure in which probes to both chromosomes are simultaneously hybridized to sperm nuclei (Han et al., 1993a). This enables accurate and unequivocal identification of the gender of each spermatozoon. Ideally, a third autosomal probe should be included to identify spermatozoa which are nullisomic for the sex chromosomes.

**Evaluation of sperm separation procedures**

**Albumin gradients**

The use of discontinuous albumin gradients for male (and female) sex pre-selection has remained controversial since the first report of Y enrichment by Ericsson et al. (1973). The controversy has been largely fuelled by conflicting evidence for Y enrichment using albumin gradients (Ericsson et al., 1973; Evans et al., 1975; Ross et al., 1975; Dmowski et al., 1979; Quinliven et al., 1982; Brandriiff et al., 1986; Ueda and Yanagimachi, 1987; Vidal et al., 1993; Wang et al., 1994a; Claassens et al., 1995). The insistence by proponents of the method that the results of uncontrolled clinical trials are of paramount importance (Beemink et al., 1993; Ericsson, 1994a,b; Pyrzak, 1994), and the fact that the hypothesized mechanisms for Y enrichment have never been experimentally tested, have further fuelled the controversy. Furthermore, the methodology is patented and its use has been limited to centres licensed by Gametrics Ltd. (Alzada, MT, USA), so it has been difficult for independent researchers to remain free from the controversy since replication of the methodology was difficult and could not be assumed (Ericsson, 1994a). Martin (1994) summarized the results on albumin gradients by stating that: ‘it has not been demonstrated scientifically that the albumin column method alters the sex ratio in spermatozoa or newborns’.

Since 1993, there have been four studies which used FISH to evaluate the claim that albumin gradients enrich Y-bearing spermatozoa. Two of these studies employed single label FISH with its attendant drawbacks (Vidal et al., 1993; Claassens et al., 1995), while the other two studies used double label FISH (Wang et al., 1994a; Flaherty et al., unpublished data). Vidal et al. (1993) modified the technique by performing the albumin gradient separations at 37°C instead of at room temperature, so their results may not be a true reflection of the licensed method. The study of Claassens et al. (1995) evaluated the protocol 3 albumin method and reported an increase in the percentage of Y-bearing spermatozoa from 50.3% in semen to 53.4% after preparation on albumin gradients. The difference was statistically significant, but obviously of no clinical relevance since it has been claimed that the method yields 75% males at birth (Beemink et al., 1993). Furthermore, the use of single label FISH precludes an accurate determination of the ratio of X- to Y-bearing spermatozoa. In contrast, Wang et al. (1994a) evaluated two albumin gradient methods, a two-step method (Dmowski et al., 1979) and Ericsson’s protocol 3 method (Beemink et al., 1993), using double label FISH. There was no enrichment of Y-bearing spermatozoa with either method, and on average a slight enrichment of X-bearing spermatozoa was found. That study was subsequently criticized by Ericsson (1994b) on a number of minor technical points, although additional supporting evidence was provided (Flaherty and Matthews, 1994).

One of the drawbacks of these FISH studies is that the licensed procedures may not have always been duplicated exactly. However, Flaherty et al. (unpublished data) recently performed the first truly objective study of albumin gradients. The study design included sperm preparation by a long-standing centre licensed by Gametrics Ltd., low recoveries of motile spermatozoa which was reported to be important for Y enrichment (Pyrzak, 1994), double label FISH analysis by an independent laboratory, and collation of results by an independent third party. Two licensed albumin procedures were evaluated, protocol 3 and modified protocol 3 (Beemink et al., 1993), but no enrichment of Y-bearing spermatozoa was found with either method. This confirmed the earlier results of Wang et al. (1994a), and showed conclusively that discontinuous albumin gradients do not enrich Y-bearing spermatozoa. The results also disagreed with the theory proposed by Pyrzak (1994) that Y enrichment occurs only if the recovery of motile spermatozoa is <10%.

Hence, evidence acquired using reliable molecular techniques to assess the sex of individual spermatozoa indicates that discontinuous albumin gradients do not enrich Y-bearing spermatozoa. The only remaining issue now is whether the reported skew in the sex ratio at birth (Dmowski et al., 1979; Beemink and Ericsson, 1982; Corson et al., 1984; Beemink et al., 1993) is merely the result of uncontrolled clinical studies. Even the extensive data presented by Beemink et al. (1993) were collated from many different licensed centres worldwide and as such were experimentally uncontrolled. The only way to clarify this issue would be to perform properly controlled clinical trials.

**Percoll gradients**

Iizuka et al. (1987) reported that discontinuous Percoll gradients could be used clinically for female sex pre-selection. They used 12-step discontinuous Percoll gradients (25–80%) and found that 94% of the spermatozoa in the 80% Percoll fraction did not stain with quinacrine and were therefore recorded as X-bearing spermatozoa. Furthermore, after artificial insemination with spermatozoa isolated on 12-step Percoll gradients, Iizuka et al. (1987) reported 6 girls born from 6 pregnancies.

Wang et al. (1994b) evaluated the 12-step Percoll gradient method using double label FISH and found that there was a slight enrichment of X-bearing spermatozoa in the 80% Percoll fraction, though considerably less than the enrichment reported by Iizuka et al. (1987). The mean X:Y ratios were 49:48 in the neat semen controls and 55:41 in the 80% Percoll fractions.
The ratios in individual Percoll fractions ranged from 51:46 to 57:38 (Wang et al., 1994b). The difference in the proportion of X-bearing spermatozoa in the studies of Iizuka et al. (1987) and Wang et al. (1994b) is quite dramatic (94% versus 55%), but the work of others clarifies this apparent discrepancy. Van Kooij and van Oost (1992) examined populations of spermatozoa isolated on 12-step Percoll gradients by Southern blot analysis using a DNA probe (pDP34) which distinguishes loci on both the X and Y chromosome and they found that there was approximately a 1:1 ratio of X- to Y-bearing spermatozoa in the 80% Percoll fraction, which is comparable to the results of Wang et al. (1994b). However, when these researchers stained the same sperm populations with quinacrine, they found that 86% of the spermatozoa did not exhibit a signal and were therefore putative X-bearing spermatozoa (van Kooij and van Oost, 1992). Therefore, it appears that isolation of spermatozoa on Percoll gradients interferes with quinacrine staining, thus yielding an artefactually elevated count of X-bearing spermatozoa. This explains why the results of Iizuka et al. (1987) were not confirmed by double label FISH analysis in which X- and Y-bearing spermatozoa were properly identified.

These studies indicate that 12-step discontinuous Percoll gradients do produce a slight enrichment of X-bearing spermatozoa; however, the level of enrichment would certainly not be sufficient to significantly influence the sex ratio at birth. The exact mechanism of enrichment using Percoll gradients is unclear (reviewed by Wang et al., 1994b).

Swim-up procedures

Check et al. (1989) reported 81% male offspring after insemination of women with spermatozoa prepared by a modified swim-up procedure, and they followed this initial report with a prospective trial in which the incidence of males at birth was 88.5% after preparation of spermatozoa by modified swim-up compared with 50% after preparation on standard Percoll gradients (Check and Katsoff, 1993). Using quinacrine staining, the incidence of putative Y-bearing spermatozoa was found to be 49.2% in the Percoll samples and 83.6% in the swim-up fractions. These data suggested that swim-up procedures can alter the ratio of X- to Y-bearing spermatozoa in the isolated motile sperm fraction and therefore could be used for male sex pre-selection. In contrast, Han et al. (1993b) examined 12 semen samples by double label FISH, both before and after isolation of motile spermatozoa using a routine swim-up procedure, and found no enrichment of X- or Y-bearing spermatozoa in the motile sperm fraction. These conflicting results might be explained by slight differences in the procedure used, such as the time of incubation (Claassens et al., 1989) or the centrifugation steps, but more likely emphasize the inadequacies of the quinacrine staining technique used by Check and Katsoff (1993). Lobel et al. (1993) also performed a study on spermatozoa prepared by swim-up using quantitative PCR to determine the X:Y ratio; they found no significant change in the X:Y ratio in the motile sperm fraction. This supports the findings of Han et al. (1993b) and further demonstrates the usefulness of modern molecular biology methods such as FISH and PCR for the evaluation of sex selection procedures.

Since there are different ways of preparing spermatozoa using swim-up techniques, further controlled studies using double label FISH will clarify whether or not X- or Y-bearing spermatozoa can be enriched by swim-up. One such controlled study has recently been completed (C.J.De Jonge and S.P.Flaherty, unpublished observations). Semen samples were prepared using a modified swim-up procedure in which putative X and Y fractions were recovered from the upper and lower regions, respectively, of the supernatant (Rawlins et al., 1988). The ratio of X- to Y-bearing spermatozoa in the undiluted semen and in X and Y fractions recovered after 15, 30, 45 and 60 min swim-ups was assessed using double label FISH. A slight enrichment (1-2%) of Y-bearing spermatozoa was found in the Y fraction after a 30 min swim-up; however, no other differences were found between controls and the X and Y fractions. It therefore appears that this modified swim-up procedure does not produce clinically significant enrichment of X- or Y-bearing spermatozoa.

Flow cytometry

Mammals exhibit inter-species differences of up to 12.5% in the total chromosomal DNA content due to differences in the size of the X and Y chromosomes (Johnson, 1994). In human spermatozoa, the difference is 2.9% (Johnson et al., 1993). After appropriate DNA staining, this difference can be exploited to separate X- and Y-bearing spermatozoa using flow cytometry (Gledhill, 1988; Johnson, 1994). This methodology has been thoroughly evaluated in a variety of animal models and can be applied to spermatozoa nuclei (Johnson et al., 1987) or to intact, live spermatozoa (Johnson et al., 1989). Yields of >90% have been achieved using spermatozoa from some species and live offspring have been produced after artificial insemination or in vitro fertilization using the sorted spermatozoa (Johnson, 1994). An advantage of this method is that the efficiency of separation of X- and Y-bearing spermatozoa can be verified by re-analysis of the separated fractions by flow cytometry (Johnson, 1988). Further information on the development, validation and application of flow cytometry can be found in reviews by Johnson (1992, 1994).

Johnson et al. (1993) reported that viable populations of human spermatozoa enriched for either X- or Y-bearing spermatozoa could be prepared using flow cytometry. They used single label FISH to verify the level of enrichment; each sorted sperm fraction was analysed twice, once using an X probe and then again using a Y probe. They found that X fractions contained 82% X-bearing spermatozoa, while Y fractions contained 76% Y-bearing spermatozoa. This was the first conclusive demonstration of a clinically-significant enrichment of X- and Y-bearing human spermatozoa, and it is possible that even higher purity fractions could be recovered using modified sorting criteria (Johnson and Schulman, 1994). Despite some concerns relating to the safety of using DNA-binding fluorochromes such as Hoechst 33342 and ultraviolet light (Ashwood-Smith, 1994; Johnson and Schulman, 1994), flow cytometry has been used successfully to increase the number of appropriate (XX) embryos available for preimplanta-
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