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SEPARATION OF SPERM THROUGH A 12-LAYER PERCOLL COLUMN DECREASES THE PERCENTAGE OF SPERM STAINING WITH QUINACRINE

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Previous methods of enriching sperm with a higher percentage of Y-bearing sperm have been questioned because the claims that Y enrichment was present were based on quinacrine staining of the Y chromosome, and the enrichment was not confirmed by polymerase chain reaction (PCR) or fluorescent in situ hybridization (FISH) techniques. A technique was evaluated that theoretically could increase the percentage of X-bearing sperm by isolating a fraction of the "heaviest" sperm by passing them through 12 layers of discontinuous Percoll gradient. Initially 12 specimens were checked both before and then after separation with 12 layers of Percoll for percentage of Y sperm. The median for baseline Y percentage was 49% and after processing the percentage of Y dropped to 10%. An additional 19 specimens were checked after separation only. The median was 19%. The sample with the lowest preseparation % of quinacrine staining sperm was 45% and the highest was 54%. After 12-layer Percoll, the lowest percentage was 3% and the highest was 24%. There have been claims that quinacrine staining can falsely increase apparent Y-bearing sperm enrichment following certain separation procedures, e.g., albumin separation, by nonspecific staining of autosomal chromosomes. If anything, then, it should falsely decrease X-bearing sperm enrichment. Thus, 12-layer Percoll separation may actually enrich for X-bearing sperm or possibly this procedure somehow nonspecifically inhibits the ability of quinacrine to stain the Y chromosome.

Keywords fluorescent in situ hybridization, Percoll separation, quinacrine, X-bearing sperm

The use of modern molecular techniques, e.g., polymerase chain reaction (PCR) or fluorescence in situ hybridization (FISH), to evaluate sex selection methods seems to invalidate previous conclusions, based on quinacrine staining of the Y chromosome, that discontinuous albumin gradients or certain swim-up techniques enrich the percentage of Y-bearing sperm [1,

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6, 9]. One explanation for the discordance is that quinacrine could sometimes nonspecifically stain autosomal chromosomes, leading to a false increase in apparently Y-bearing sperm [2]. Some researchers question this conclusion and state that differences in methodology, especially for the swim-up procedures, could account for the differences [3, 4].

In studies using FISH technology, the efficacy of an enriching technique using 12-layer discontinuous Percoll gradients was shown by a mild enrichment of X sperm (55.1:41.1 or 1.35), but the degree of enrichment was insufficient for use in preconceptional sex selection [10].

As yet, no mechanisms have been proposed as to how separation procedures could prevent staining of the Y chromosome by quinacrine to give falsely increased percentage of X-enriched sperm. This study was originally intended to simultaneously compare the percentage of X- and Y-bearing sperm following separation by a 12-layer discontinuous Percoll gradient as determined by quinacrine staining vs. FISH. Unfortunately, unforeseen events precluded the evaluation by FISH, but the quinacrine data are provided.

MATERIALS AND METHODS

12-Layer Percoll Density Gradient

Columns were prepared using 15-mL conical, sterile centrifuge tubes. Layers were made using isotonic Percoll supplemented with 0.5% human serum albumin. The medium was a modified human tubal fluid (HTF) with 0.5% serum substitute added as the protein source. Then 0.5-mL layers of Percoll:HTF were overlaid, beginning with 80% in 12 steps to end with 25%. The liquefied semen was diluted with an equal part of medium, and up to 2 mL were overlaid on top. Columns were centrifuged at 250*g* for 30 min. The sperm pellet was then removed and washed twice.

Quinacrine Staining

A 0.1% solution of quinacrine mustard and cold distilled water was made fresh just prior to testing. A wet preparation of 7 μL of stain was mixed on a glass slide with 7 μL of the washed sperm specimen. The slides were read immediately using fluorescent microscopy. Sperm heads containing a fluorescent round body were counted as Y-bearing sperm. As a stain control, a raw semen sample was counted for 50 ± 2% Y-bearing sperm.

RESULTS

Initially 12 specimens were checked both before and after separation with 12-layer Percoll for % Y sperm. The median for baseline Y percentage was 49% and after processing the percentage of Y dropped to 10%. An additional 19 specimens were checked after separation only. The median was 19%. The sample with the lowest preseparation percentage of quinacrine staining sperm was 45% and the highest was 54%. After 12-layer Percoll separation the lowest percentage of sperm staining positive with quinacrine was 3% and the highest was 24%.

DISCUSSION

The fact that 5 andrologists consistently observed a decreased percentage of sperm staining positive with quinacrine suggests that the data are not biased by the somewhat difficult detec-

tion of the quinacrine-stained specimen under fluorescent microscopy. The degree of X-bearing sperm enrichment, as determined by quinacrine staining, using a 12-layer Percoll separation procedure was much higher than reported by Wang et al. using FISH for evaluation [10]. There are several theories to explain the discordant results. One possibility is that passage of sperm through many Percoll layers somehow nonspecifically prevents quinacrine staining of the Y chromosome. A second possibility is that somehow the separation procedure inhibits binding of the X probe when measured by FISH. A third possibility is that there are some subtle differences in the 12-layer Percoll method between the two centers, and that, had we been able to use evaluation by PCR or FISH, a greater X enrichment might have been found than reported previously [10].

The use of MicroSort flow cytometry has apparently been successful in enriching sperm, especially for X-bearing sperm, as determined by FISH [5, 7, 8]. This is an expensive procedure and sometimes the sperm yield is poor, requiring in vitro fertilization with intracytoplasmic sperm injection. Thus, it is important to once and for all determine whether the 12-layer Percoll separation procedure, at least as performed at our center, provides only a mild X enrichment similar to the report of Wang et al. [10] or provides enrichment similar to what we are estimating by quinacrine staining. Thus, we hope that we can conduct another series where we can evaluate the percentage of X and Y sperm using modern molecular techniques in addition to quinacrine and compare the differences.

The only way to completely answer the possibility that the 12-layer Percoll separation may falsely lower the percentage of apparently Y-bearing sperm is to compare sex gender outcome in sperm separated using this technique vs. the MicroSort procedure. If it is proven that the 12-layer Percoll separation yields false decrease in staining of the Y chromosome, it would be of interest to determine why a swim-up procedure would falsely increase staining of the Y chromosome and a Percoll method falsely decrease staining of the Y chromosome.

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