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# A prospective study to evaluate the efficacy of modified swim-up preparation for male sex selection

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**A previously published study reporting the use of a modified swim-up technique for sperm preparation prior to insemination which resulted in a high percentage of male births has been criticized for its lack of controls. The present prospective study was initiated to investigate further the efficacy of modified swim-up preparation for male sex-selection when applied in a properly defined control group. Our results showed that the proportion of males born in singleton pregnancies was ~50% in the group inseminated following sperm preparation with Percoll and in the control group with no sperm preparation compared with 88.5% in the group treated with the modified method of swim-up sperm preparation prior to insemination. This high rate of males in the group treated with modified swim-up was also observed in singleton pregnancies of women taking ovulation inducing drugs (primarily clomiphene citrate). This contrasts with previous publications in which a higher rate of females was found in clomiphene citrate patients using the albumin separation technique. How the mechanism of the swim-up procedure may result in a high male birth rate remains unclear. A high percentage of y enriched semen was found using fluorochrome quinacrine mustard staining but this procedure may falsely stain autosomal chromosomes. If analysis using sensitive DNA probes fails to confirm the y enrichment of the spermatozoa, one must hypothesize that the modified swim-up procedure damages the x-spermatozoa.**

*Key words:* fluorochrome quinacrine staining/male sex selection/ Percoll/prospective study/swim-up

## Introduction

Several methods claim to enrich the semen specimen for y-bearing sperm. One in particular is the albumin separation technique, which uses a column with either two or three layers of human serum albumin (Ericsson *et al.*, 1973). One study revealed a concentration of 85% y-bearing sperm in the higher albumin fraction (20%) as determined by quinacrine staining of the long axis of the y chromosome (Zech *et al.*, 1969). A male birth rate of 73.5% (39 males, 16 females) was reported using the three layer technique, but when the females were excluded from taking clomiphene citrate, there was a male birth ratio of 82.6% (38 males and nine females) (Beernink and Ericsson, 1982). Thus using the same technique, but placing the female partner on clomiphene, a 7:1 female/male ratio would be achieved.

Two very similar swim-up procedures claim to increase the percentage of male offspring and increase the percentage of y-bearing spermatozoa (Rawlins *et al.*, 1988; Check *et al.*, 1989). Rawlins *et al.* (1988) found that pregnancies achieved by in-vitro fertilization (IVF) with spermatozoa prepared by the swim-up method resulted in 10 males delivered versus one female (90.9%). Check *et al.* (1989) studied in-vivo pregnancies and reported 86% male deliveries following insemination after spermatozoa were separated by the swim-up method described; even more interesting, 10 women were treated with human menopausal gonadotrophins (HMG) and nine with clomiphene, with a result that 19 of 22 births were males (Check *et al.*, 1989).

Several criticisms have been documented in regard to the studies of male pre-selection. The fluorescent 'y-body' acrosin technique may falsely increase the y-bearing sperm count due to nonspecific fluorescence of autosomal chromosomes (Carson, 1988). One study evaluated the albumin separation technique for y-enrichment, not by quinacrine fluorescence, but with a modification of the zona-free hamster-egg penetration test designed to obtain sperm karyotypes. This technique failed to confirm enrichment for y-bearing spermatozoa; in fact, a slight predictor for x-bearing spermatozoa was found (Brandriff *et al.*, 1986).

One of the main criticisms of the previous studies was the lack of controls; also small numbers in the study groups may account for the fortuitous skewing of data. Furthermore, those centres not getting the desired ratio of males may simply not report their results. As noted by Begg and Berlin (1988), publication bias is a serious problem in the intepretation of scientific research.

The study presented herein was a randomized and prospective controlled evaluation to examine the outcome of the modified swim-up preparation to see if a higher ratio of male births would occur. Furthermore, the study evaluates the use of ovulation inducing drugs (OVID) in altered higher male birth ratios.

## Materials and methods

In this study there were two treatment groups and one control group. The first treatment group consisted of patients for whom the modified method of swim-up sperm preparation was used prior to insemination, specifically for male selection. In the

modified swim-up procedure, each semen sample was first mixed with an equal volume of Ham's F-10 medium and 0.5 ml portions of the mixture were placed in 1.5 ml microcentrifuge tubes. The tubes were then layered with 0.5 ml of Ham's-F10 and subjected to centrifugation at 500 *g* for 5 min. This was followed by a 1 h incubation at 37°C in 5% $CO_2$. The supernatant was then diluted 1:1 with medium and centrifuged for 5 min. The resulting supernatant was discarded, and the pellet was suspended in 1 ml of medium and again subjected to centrifugation. The final pellet was suspended in 0.2 ml of medium for intrauterine insemination (IUI) or 0.5 ml of medium for intracervical insemination (ICI). This treatment group comprised patients coming to our practice for male selection within a 2-year period, none of whom had infertility problems.

The second treatment group consisted of patients who received the Percoll discontinuous density gradient method (Iizuka *et al.*, 1988) of sperm preparation prior to IUI for the treatment of cervical factor. For this treatment, a modified Iizuka's four layer procedure was used. The semen was diluted 1:1 with Hank's solution. The layers were 1.0 ml of successively 80, 70, 60 and 50% Percoll. Up to 2.0 ml of the diluted semen was layered on top, then centrifuged for 20 min at 500 *g*. The top semen layer was removed and discarded. The 70, 60 and 50% Percoll layers were combined and washed twice with Hank's solution. The final pellet was resuspended to 0.5 ml for intracervical insemination. Approximately 0.75 ml of the bottom 80% Percoll layer was removed, washed twice with Hank's solution and resuspended to 0.2 ml for intrauterine insemination. This technique was used because it provides our infertility centre with a high pregnancy rate following IUI.

Patients in this group consisted of infertile women who required a sperm preparation procedure for IUI because of a cervical factor problem (some cervical factor problems occurred because of treatment of ovulation problems with clomiphene citrate). This group was not interested in sex selection. Since there are many more patients treated for cervical factor than sex selection, we did not include all cervical factor patients, but we prospectively matched one individual with cervical factor with each male selection patient. The criteria for matching was the date pregnancy was achieved. Once a sex-selection patient achieved a pregnancy, she was matched with the first cervical factor patient treated with Percoll and IUI to attain a positive beta human chorionic gonadotrophin ($\beta$-HCG).

The control group received no sperm preparation, since in all cases insemination was natural. Patients in this group were selected from the population of infertile women treated in our practice, most of whom had problems with ovulation. The patients selected for inclusion in this group were matched to sex-selection patients by date of first positive $\beta$-HCG.

All inseminations in this study involved husband's spermatozoa, therefore no donor sperm was used. Fluorochrome quinacrine mustard staining was used to evaluate the percentage of y-body spermatozoa in the sample after sperm preparation and prior to insemination. The volume, count and motility of all semen samples were recorded before and after sperm preparation so that the total amount of motile spermatozoa and percentage recovery of motile spermatozoa after sperm preparation could be compared.

**Table I.** Distribution of singleton births by gender and treatment group

| | Male | Female |
|---|---|---|
| Group 1: Modified swim-up | 23 (88.5%) | 3 (11.5%) |
| Group 2: Percoll | 12 (57.1%)[a,b] | 9 (42.9%) |
| Group 3: No sperm preparation | 12 (54.5%)[c,b] | 10 (45.5%) |

[a] $\chi^2 = 6.0$, df = 1, $P = 0.01$ comparing group 1 to group 2.
[b] $\chi^2 = 0.3$, df = 1, $P = 0.86$ comparing group 2 to group 3.
[c] $\chi^2 = 6.9$, df = 1, $P = 0.01$ comparing group 1 to group 3.

The outcome of each pregnancy was recorded with regard to number of babies born (singleton, multiple) and gender of all live births. The proportion of male births in singleton and multiple pregnancies was computed and compared by sperm preparation method using chi-square analysis. The relationship of sperm preparation to gender of baby controlling for the administration of OVID therapy was analysed using logistic regression. The level of significance used was $P < 0.05$.

## Results

By fluorochrome quinacrine mustard staining, 83.6% y-body spermatozoa were found prior to insemination in samples prepared with modified swim-up versus 49.2% y-body spermatozoa in samples prepared with Percoll. Following preparation with modified swim-up, the average total number of motile spermatozoa was $26.6 \times 10^6$/ml with an average of 17.8% recovery of motile spermatozoa. Following preparation with Percoll, the average total number of motile spermatozoa was $10.6 \times 10^6$/ml with an average recovery of 26.1% motile spermatozoa.

Spontaneous abortions occurred in three women in treatment group 1, two from treatment group 2 and eight controls. Thus, the number of patients with live births evaluated in each group was 26 in group 1, 21 in group 2 and 23 in group 3.

Sixty-nine of the 80 pregnancies resulted in singleton births. The distribution of singleton births by gender and treatment group is presented in Table I. The proportion of male births in the group treated with swim-up (88.5%) was significantly higher than that found in the Percoll group (57.1%) (chi-square, $P = 0.02$) and that found in the control group (54.4%) (chi-square, $P = 0.01$). There was no difference in the proportion of male births that occurred in the Percoll and control groups (chi-square, $P = 0.86$).

Multiple births occurred in 11 pregnancies; all of these women had been treated with ovulation inducing drugs. The distribution of multiple births by gender and treatment group is presented in Table II. Eight of the 11 multiple births were bi-sexual, and three were uni-sexual. Although overall 81.8% of the multiple births had at least one male, the proportion of males computed for all babies born in multiple births according to treatment was 50% for group 1; 41.2% for group 2 and 33.3% for group 3. There was not a sufficient number of multiple births in each treatment group to make any statistical inferences, but the data for the modified swim-up group appears to differ from that found in singleton pregnancies and thus it was felt that the data from single and multiple births should not be pooled for further analysis.

**Table II.** Distribution of multiple births by gender and treatment groups

| | Group 1 Swim-up | Group 2 Percoll | Group 3 No sperm preparation |
|---|---|---|---|
| Twins: M,M[a] | 0 | 1 | 0 |
| Twins: F,F | 0 | 2 | 0 |
| Twins: M,F | 2 | 4 | 0 |
| Triplets: M,F,F | 0 | 1 | 1 |
| Total no. of infants | 4 | 17 | 3 |
| Proportion of males | 50% | 41.2% | 33.3% |
| Proportion of multiple births with at least one male | 100% | 75% | 100% |

[a]M = male, F = female.

**Table III.** Distribution of singleton births by gender, treatment group and administration of ovulation inducing drugs (OVID)

| | No OVID | | OVID | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| Group 1: Swim-up | 10 (83.3%) | 2 (16.7%) | 13 (92.8%) | 1 (7.2%) |
| Group 2: Percoll | 3 (50.0%) | 3 (50.0%) | 9 (60.0%) | 6 (40.0%) |
| Group 3: No sperm preparation | 3 (50.0%) | 3 (50.0%) | 9 (56.2%) | 7 (43.8%) |

The data from the singleton pregnancies were further analysed to see if the relationship observed between sperm preparation and gender was the same for these patients receiving OVID therapy compared to those not receiving OVID therapy. The distribution of singleton pregnancies by gender, treatment group and OVID received is presented in Table III. The data were fitted to a logistic regression model in which the gender outcome was the dependent variable, and sperm treatment and OVID therapy were the independent (explanatory) variables. The regression coefficients for each variable are presented in Table IV. The coefficient for OVID was not significantly different from zero, thus the gender of the baby was not related to the OVID therapy received. The only variable found to have a significant relationship to the gender of the baby was treatment with modified swim-up.

**Table IV.** Results of the logistic regression analysis to predict gender outcome

| Variable | Coefficient | Standard error | 95% confidence interval |
|---|---|---|---|
| Swim-up[a] | 1.93* | 0.77 | (0.42, 3.44) |
| Percoll[b] | 0.12 | 0.62 | (−1.09, 1.33) |
| OVID[c] | 0.29 | 0.61 | (−0.91, 1.48) |

[a]Coded as 1 for patients using swim-up, 0 for all others.
[b]Coded as 1 for patients using Percoll, 0 for all others.
[c]Coded as 1 for patients receiving ovulation inducing drugs, 0 for those not receiving therapy.
*$P < 0.05$.

## Discussion

After testing various sperm separation techniques to see if we could influence the proportion of y- or x-bearing spermatozoa, we found the modified swim-up to produce a high enrichment of y-bearing spermatozoa, at least as determined by fluorochrome quinacrine mustard staining. The claim is made concerning the albumin separation technique that a high delivery rate of males will occur, but if a female is desired the same technqiue is used, with the exception of treating the women with clomiphene (Beernink and Ericsson, 1982). Our original study was intended to compare the percentage of male births using the modified swim-up in 10 clomiphene treated patients versus 10 HMG treated women (Check *et al.*, 1989). One patient was lost in follow-up, therefore birth information was available for 10 HMG and nine clomiphene treated women. In contrast to the albumin separation technqiue, a high rate of male infants was found in clomiphene or HMG treated women.

Our previous study comprised infertility patients who were not seeking sex pre-selection. In fact, they were only included if they answered the questionnaire stating they had no preference for gender outcome. In contrast for the study presented herein, group 1 was composed of patients seeking male selection. They were offered either the albumin separation technique or the modified swim-up, but all chose the latter.

We wanted to use a control to exclude the possibility that any sperm separation procedure followed by IUI might result in a higher percentage of male births. For that reason we chose patients requiring IUI for cervical factor. Unfortunately, we did not realize this group would have a much higher percentage of patients taking clomiphene. Thus, one could argue that the data are still consistent with the hypothesis that any separation procedure followed by IUI leads to a high rate of male births except when the female has taken clomiphene, which brings the ratio closer to 50%. However, against this hypothesis is the finding that in group 1 patients, those treated with clomiphene also had a high rate of male births. Furthermore, in group 3, 13 of 18 patients taking OVID took clomiphene, thus representing over half of the patients in the group, and no skewing toward females occurred despite none of them having IUI.

A possible flaw of the study using the IUI control group (group

2) was that a discontinuous Percoll density gradient was used for separation. Kaneko *et al.* (1984) have described a Percoll separation method which resulted in an 82% x-bearing bottom layer when an eight layer gradient was used and a 94% x-bearing bottom layer when the number of layers was increased to 12. Our Percoll method, however, used only four layers (50, 60, 70 and 80%) and therefore it is not known if an enriched concentration would also be realized. Proper randomized studies have never been performed to substantiate a higher percentage of female births with the Percoll technique (Carson, 1988). Furthermore, no x-enrichment was found following this four layer technique.

Actually, if there was any selection bias against any one group for male selection it would be group 1. Whereas the majority of group 2 and 3 patients had primary infertility, in the 14 with secondary infertility who had previously delivered a baby, there were eight males and six females. In contrast, there were 63 females born out of 68 babies to group 1 patients prior to this study. Thus the possibility existed that certain males, for unknown reasons, produce a high rate of females; perhaps there exists some x-linked lethal condition resulting in the early abortion of males. Thus the high rate of male babies in this group is certainly very impressive.

We were aware at the time of this study that the quinacrine technique of staining for fluorescent y-body was considered falsely to increase the percentage of y. Unfortunately, we did not have access to be able to perform the more sensitive DNA-probing technique (Zech *et al.*, 1969). There was only a 50% incidence of y-bearing spermatozoa using the quinacrine method when the sperm was prepared by discontinuous Percoll gradient. If one hypothesizes a false increase in y because of staining of abnormal chromosomes then the sperm preparation from group 2 males would have to have been skewed toward female enrichment; yet no higher ratio of female births was found.

Our original study was performed in two different states (Pennsylvania and Indiana) by two different groups and both found a high percentage of male births (Check *et al.*, 1989). Rawlins *et al.* (1988) using a similar technique found a high incidence of male offspring from in-vitro fertilization–embryo transfer (IVF–ET) conceptions. This study confirmed our previous findings and compared controls matched in a prospective manner. The procedure is inexpensive, simple to perform and produces better quality spermatozoa than the non-separated specimen. Nevertheless, we still believe that the data should be confirmed by another group before recommendations for widespread use of the modified procedure be made.

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