Discontinuous Percoll gradient preparation for donor insemination: determinants for success

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The use of cryopreserved donor spermatozoa for insemination has become necessary to decrease the risk from sexually transmitted infectious diseases. Lower fecundity rates have been reported with this practice. Efforts have been applied to increase success, including identification of those sperm characteristics which correlate with increased fecundity. Data from in-vitro fertilization have revealed sperm morphology, motility and zona pellucida binding as important sperm parameters. Discontinuous Percoll gradient preparation yields a high concentration of motile spermatozoa. Using this preparation for thawed donor spermatozoa, we have identified post-preparation motility and progression as factors associated with increased fecundity. Consideration should be given to screening sperm donors with a freeze-thaw Percoll gradient preparation prior to acceptance into a donor bank.

Key words: donor sperm/fecundity/intrauterine insemination/ Percoll gradient/sperm motility

Introduction

Rates of conception with artificial insemination by donor (AID) using fresh donor spermatozoa approximate those in normal fertile couples (Meeks et al., 1986; Hassiakos et al., 1990). However, to lessen the risk of sexually transmitted diseases, use of cryopreserved–thawed donor spermatozoa has become almost universal. Lower fecundity rates have been reported with this practice (Subak et al., 1992).

Investigations to identify factors related to success rates have examined a number of variables. Intrauterine insemination rather than intracervical placement has become widespread (Byrd et al., 1990; Patton et al., 1992). Jeyendran et al. (1995) recommended the use of TEST–yolk media, both as a cryoprotectant to allow greater retention of post-thaw motility and as an efficacious medium for sperm processing for intrauterine insemination. Other factors examined include recipient age (Peters et al., 1993; Kang and Wu, 1996) and reproductive history (Kovacs et al., 1982; Chauhan et al., 1989), indication for AID (Smith et al., 1981; Kovacs et al., 1982; Hammond et al., 1986), frequency and timing of insemination (Brook et al., 1994; Khalifa et al., 1995) and freezing technique (Prins and Weidel, 1986).

Data from in-vitro fertilization suggest normal sperm morphology and motility, and zona pellucida binding, as the sperm parameters most closely correlated with increased fertilization rate and pregnancy (Liu and Baker 1992). Evaluation of intrauterine insemination cycles using fresh partner’s spermatozoa has pointed to post-wash total motile count (Huang et al., 1996) and normal morphology (Burr et al., 1996) as the sperm variables most affecting fecundity. Recently, Johnston et al. (1994) undertook a 15 year retrospective analysis of intracervical inseminations using thawed spermatozoa without special preparation. The parameters post-thaw motility, morphology and sperm count were found to be the most important factors for the selection of fertile donors.

Intrauterine placement of donor spermatozoa is known to increase fecundity and has replaced intracervical inseminations in many clinical settings. Various methods of sperm preparation have been described in an effort to increase the recovery of high quality spermatozoa as indicated by increased motility. Comparing swim-up, albumin gradient and one-step discontinuous Percoll gradient, Berger et al. (1985) noted that the discontinuous Percoll gradient yielded a highly motile homogeneous sperm preparation with increased penetration of zona-free hamster ova. The present prospective study was undertaken to identify seminal parameters associated with increased pregnancy rates in cycles of intrauterine inseminations (IUI) utilizing cryopreserved donor spermatozoa prepared using a discontinuous Percoll gradient.

Materials and methods

All intrauterine insemination cycles using cryopreserved donor spermatozoa were examined during the period September 1991 through December 1995. Only those cycles with a single insemination were included. Specific factors for each cycle were recorded, including recipient age, pregnancy history, concomitant infertility factors, indication for AID, use of ovulation induction agents and luteal phase support and pre- and post-preparation seminal parameters.

All patients undergoing AID and donors were screened for infectious diseases according to the revised American Society for Reproductive Medicine guidelines (American Fertility Society, 1991). Timing of insemination was based on detection of the luteinizing hormone (LH) surge with urinary LH kits, with IUI performed the day after a positive test or 36 h after administration of human chorionic gonadotrophin (HCG). In the absence of a positive history for tubal disease, tubal assessment was done by hysterosalpingography after the third cycle. Laparoscopy was employed when adhesive

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disease or endometriosis was considered. Patients aged >35 years were screened for basal FSH concentrations.

Semen samples were collected after a 2–3 day period of abstinence. After liquefaction, the sample was transferred to a 15 ml centrifuge tube and diluted with TEST–yolk buffer with glycerol (Irvine Scientific, CA, USA) until a 1:1 sample to cryoprotectant ratio was achieved. The mixture was placed in a 37°C waterbath for ~10 min. Total volume was recorded and 15 µl of the sample was evaluated for sperm concentration, progression, motility and total motile count prior to freezing. Analysis was performed manually by a limited number of technicians according to standard techniques and keeping inter-observer error to a minimum. The remaining sample was then refrigerated at 2°C to allow slow cooling of the mixture. After 90 min the semen mixture was separated into 1 ml straws and cryopreserved in a step-wise fashion using a Nicool (Compagnie Francaise de Products Oxygenes, Sassenage, France) LM-10 control rate freezer (Prins and Weidel, 1986).

Preparation for the 85/40% discontinuous Percoll gradient was undertaken to achieve the appropriate concentrations as outlined in Table I. The reagents were mixed in sterile flasks and filtered through a 0.2 µm Nalgene filter. The prepared Percoll was then aliquoted into 5 ml samples for storage.

Spermatozoa for AID/IUI were prepared by thawing individual straws in a 37°C waterbath for 10 min. Total volume was recorded and a 15 µl portion was evaluated to determine sperm concentration, progression, motility and total motile count. The sample was then layered on top of a discontinuous Percoll (Sigma, St. Louis, MO, USA) gradient, which consisted of 0.5 ml of 85% Percoll overlaid with 0.5 ml of 40% Percoll, and was immediately centrifuged for 30 min at 300 g. The sperm pellet was then washed twice with 0.75% human serum albumin (HSA) in Ham’s F-10 medium (Gibco, Grand Island, NY, USA). The supernatant was removed to within 0.2 ml of the sperm pellet. The pellet was resuspended in 3% HSA 25% in Ham’s F-10 to a total volume of 0.5 ml. Examination of the specimen for insemination was performed to note post-preparation concentration, motility, progression and total motile count.

The sample was drawn up into a 1 ml syringe and attached to a pre-rinsed Tomcat (Sherwood Medical, St. Louis, MO, USA) or Shephard (Cook OB/GYN, Spencer, IN, USA) catheter. After wiping the exocervix, intrauterine placement of the spermatozoa was performed.

Pregnancy was detected by the presence of successively rising serum HCG values but only those showing a gestational sac on vaginal ultrasonography were considered for statistical analysis.

Comparison of variables between the pregnant and non-pregnant groups was performed by application of the χ² test, Cochran’s test for linear trends in proportions and analysis of variance as appropriate. Data for life table analysis for first pregnancy were compiled as described by Kaplan and Meier (1958).

Results
In all, 143 women underwent 796 cycles of single IUI with thawed donor spermatozoa. A total of 112 clinical pregnancies occurred in 76 of these patients, resulting in a 53.1% pregnancy rate per patient. There were 76 pregnancies achieved in 581 cycles of first pregnancy attempts (13.1% cycle fecundity), 28 pregnancies in 152 cycles of second pregnancy attempts (18.4% cycle fecundity), seven pregnancies in 54 cycles of third tris (13.0% cycle fecundity) and one pregnancy in nine cycles of fourth pregnancy attempts. Overall cycle fecundity equalled 14.1%.

A Kaplan–Meier Survival Curve was constructed for first pregnancy cycles (Figure 1). The 50% cumulative pregnancy mark was reached at the sixth cycle and no pregnancies occurred after the twelfth cycle. There was an 86.9% theoretical cumulative pregnancy rate reached at the twelfth cycle. For those conceiving, the mean and median time to pregnancy are shown in Table II. Cycle fecundity for the first six cycles in first pregnancy attempts was 12.5, 15.7, 8.6, 8.0, 11.3 and 19.0% respectively. The variation was not statistically significant.

Of conceiving patients, 85% had a single intrauterine pregnancy noted by ultrasound, 9% had twins and the remainder had a higher order multiple gestation. There were no ectopic pregnancies in this series.

The mean age of patients was 34.8 (± 4.5) years, with minimum and maximum values of 24 and 46 respectively. Fifty-three percent of these patients had never been pregnant, 21.3% had been pregnant but had no resultant live birth and the remainder of patients had experienced a live birth. Indications for AID included a male partner with azoospermia (42.9%), oligozoosperma (26.5%), or a genetic factor (1.4%). Patients with no male partners comprised 22.6% of the sample, with the remaining 6.6% having ‘other’ indications. Concomitant female infertility factors included ovulatory dysfunction (42.5%), endometriosis (12.9%), mild tubal disease (6.9%) and uterine factor

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<th>Table I. Percoll preparation for the 85/40% discontinuous gradient</th>
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<td>Final concentration</td>
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<td>85%</td>
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<td>10× Ham’s F10 (ml)</td>
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<td>H₂O (ml)</td>
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<th>Table II. Median and mean time to pregnancy</th>
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<td>Pregnancy</td>
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Figure 1. Life table analysis—first pregnancy, showing a Kaplan–Meier cumulative pregnancy curve.
To determine the impact of semen parameters on fertility, 402 cycles were studied. These cycles were divided into those with values between 0 and 3.5, and those with values between 3.5 and 4.0. The linear trend of increasing pregnancy rate with increasing total motile count was significant at \( P = 0.0066; \text{Table IV} \). Sperm progression was ranked on a scale from 0 through 4. Cycles were divided into those with values <3.5, equal to 3.5 and >3.5. Pre-Percoll-prepared sperm progression was of no prognostic significance. For post-Percoll progression, fecundity rates were 9.8, 12.6, 13.5 and 24.7% respectively. The linear trend in these pregnancy rates was statistically significant (\( P < 0.001 \)).

Multiple logistic regression was used to assess the substantive importance of the associations between semen parameter values and the pregnancy rate. Odds ratio and 95% confidence intervals are presented in Tables VI and VII for post-Percoll-prepared motility and progression with pregnancy respectively. The crude odds ratio comparing cycles with post-Percoll motility of between 81 and 95% to cycles with values <60% was 3.00. Similarly, the odds ratio comparing cycles with post-Percoll
motility values of between 75 and 80% to those with values <60% was 1.4, and for values between 60 and 75% the odds ratio was 1.3. Overall, the post-Percoll motility factor was statistically significant ($P = 0.001$).

The odds ratio comparing post-Percoll-prepared sperm progression of between 3.5 and 4.0 to progression of <3.5 was 1.7. The odds ratio comparing cycles with post-Percoll progression values equal to 3.5 to those with values <3.5 was 1.1. Overall, post-Percoll progression was statistically significant ($P = 0.03$).

**Discussion**

Anonymous donor sperm programmes are virtually limited to cryopreserved samples. This series displays an average cycle fecundity of 14.1%, a first pregnancy rate of 53.1% and a cumulative pregnancy rate for achievement of first pregnancy calculated to be 86.9% by the twelfth month. Close examination of cycle fecundity and life table analyses reported with the use of fresh donor spermatozoa in many studies reveals little difference between fresh and frozen spermatozoa. Using fresh spermatozoa, Meeks et al. (1986) reported a crude pregnancy rate of 58% with average fecundity of 15.1%. Cumulative probability of conception was reported to be 97.1% at the end of 12 cycles. In 1987, Yeh and Siebel reported a 54% pregnancy rate and 9.9% pregnancy per insemination cycle in fresh donor sperm cycles. The 50% cumulative pregnancy mark was reached in the fourth month. Similar results are noted throughout the literature on fresh donor insemination (Strickler et al., 1975; Smith et al., 1981; Keel and Webster, 1989). Examination of the only randomized, crossover design series comparing fresh and frozen spermatozoa noted a reduced cycle fecundity when a cervical insemination technique was used, but not when intrauterine insemination took place (Subak et al., 1992). The life table analyses of that series of 57 women reported a 48 ± 10% for fresh versus 22 ± 8% for frozen–thawed spermatozoa at a limited three cycle point. These values suggest that the only real difference in pregnancy rates achieved with fresh compared with frozen spermatozoa was rapidity rather than eventual success. The 50% cumulative pregnancy rate was achieved at 6 months in our series, in contrast to ≥3 months in reports where fresh spermatozoa were used. Although the risk of human immunovirus (HIV) transmission, albeit remote (Centers for Disease Control, 1988), leaves no choice regarding use of fresh spermatozoa, the differences concerning length of time to conception have relevance for patient counselling, financial considerations and future study design.

Our results do not support previous work that suggests diminished fecundity as a result of increasing recipient age (Federation CECOS, 1982; Peters et al., 1993; Kang and Wu, 1996), indication for AID (Smith et al., 1981; Kovacs et al., 1982; Hammond et al., 1986), and comitant fertility factors (Kovacs et al., 1982). This discordance may be explained in part by the differences in patient population and number, differences in screening and initial work-up, identification of concurrent diagnoses, insemination techniques, sperm preparation and ultrasound-diagnosed pregnancy rather than biochemical pregnancy. Success of AID with cryopreserved spermatozoa is influenced by the site of sperm deposition (Byrd et al., 1990; Patton et al., 1992), correct timing (Pedersen and Lebech, 1971; Marshburn et al., 1992) and sperm quality. Efforts to select fertile donors based on history and conventional sperm parameters have been inadequate (Johnston and Kovacs, 1994). Using pre-freezing computer-aided semen analysis (CASA), Marshburn et al. (1992) demonstrated curvilinear velocity, straight line velocity and total number of motile spermatozoa inseminated to be the most significant predictors of fertility following cryopreserved, intracervical inseminations.

Using CASA, Kinzer et al. (1995) described motility characteristics in sperm populations from fresh and cryopreserved semen after discontinuous Percoll gradient preparation. Cryopreservation led to a lower number of motile spermatozoa, decreased curvilinear velocity (VCL) and decreased amplitude of lateral head displacement (ALH) when compared with the original population. When studied over time, these differences were lost at 4 h because of dwindling motility, VCL and ALH in the fresh spermatozoa, while the Percoll-prepared cohort maintained the characteristics apparent immediately after preparation for up to 48 h.

Our data suggest that thawed sperm motility after discontinuous Percoll gradient preparation is prognostically related to fertilizing capability. Although much is unknown regarding the impact of freeze–thaw on spermatozoa, maintenance of a high degree of motility may reflect resistance to the ultrastructural breaks seen in cryopreserved spermatozoa (Pedersen et al., 1971). Today, it is common for donors to undergo a freeze–thaw trial prior to acceptance into a donor bank. Given the cost and expectations associated with AID and the present evidence of higher fecundity associated with higher motility rates, consideration should be given to screening all donors with a freeze–thaw Percoll preparation trial.

Finally, many AID programmes set guidelines for a lower limit of total motile spermatozoa to be inseminated. We could not correlate final number of spermatozoa or total number of motile spermatozoa inseminated with achievement of pregnancy. Sperm motility, and progression to a lesser extent, were the only important parameters.

**References**


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