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Artificial insemination and in-vitro fertilization using donor spermatozoa: a report on 15 years of experience

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Donor insemination (DI) using cryopreserved semen commenced at The Royal Women's Hospital in 1976. Over the next 15 years we performed 5953 treatment cycles to achieve 816 pregnancies (13.7% per cycle) and 706 live births. In-vitro fertilization (IVF) using donor spermatozoa commenced in 1986. Over the next 5 years we performed 303 treatment cycles for 185 couples. Including subsequent transfer of cryopreserved embryos, a total of 33% of couples achieved a successful pregnancy by IVF. Statistical analysis indicated that, for DI pregnancies, the most important semen variable was the percentage post-thaw motility, whilst for normal fertilization in IVF it was the pre-freeze motility. These results may be explained by the compensatory effects of post-thaw processing of spermatozoa for IVF, but not for DI in our clinic.

Key words: cryopreservation/donor insemination/in-vitro fertilization

Introduction

Donor insemination (DI) using cryopreserved semen was facilitated by the discovery in 1949 of the cryoprotective properties of glycerol (Polge *et al.*, 1949). Subsequent work showed that enhanced motility recovery could be obtained in the presence of additional components such as egg yolk and glycine (Sawada, 1964). Cooling rates ranging from 1 to 375°C/min have been used successfully to freeze human semen (Kremer *et al.*, 1987; Paz *et al.*, 1990). Optimal motility recovery depends on complex interactions between cryoprotectant concentration, cooling rate, warming rate and the post-thaw environment in which the sperm motility is evaluated. There is also considerable individual variation: motility recovery values of 40–70% are commonly obtained using semen from selected donors. It has been estimated that in the last 40 years >50 000 children have been conceived following DI using cryopreserved semen (see below). The incidence of spontaneous abortions and birth defects is no higher than that for the general population (Sherman, 1986).

A total of 767 live births have occurred following DI or in-vitro fertilization (IVF) using donor spermatozoa at this Hospital (1976–1991). This report describes our methods and presents a detailed analysis of our more recent results (1986–1991). The aim of this study was to determine, from retrospective analysis, which donor-related semen factors were associated with the successful use of cryopreserved spermatozoa in DI and IVF.

Materials and methods

All inseminations were performed by nursing sisters in the Reproductive Biology Clinic of this Hospital. Only four nurses have been in charge of this clinic since 1976.

Semen analysis

Semen analyses were performed manually according to the World Health Organization (WHO) guidelines (1980) current at that time. An acceptable semen analysis for a donor usually required a count of $\geqslant 50\times10^6$ spermatozoa/ml, motility of ⩾50% and <70% abnormal forms. However, occasionally donors were accepted with semen results outside these ranges if spermatozoa showed good post-thaw recovery.

Donor selection

Donors were provisionally accepted into the programme if they were between 18 and 40 years old, had no personal or family history of genetically transmittable disease, no evidence of venereal disease, had acceptable semen analysis, and had no detectable sperm antibodies by direct immunobead test (Clarke *et al.*, 1985). All donors accepted into the programme had a medical examination and since 1985 all donors have also been counselled by a social worker about the socio-legal aspects of semen donation. Final acceptance as a donor depended on the outcome of a freeze–thaw test on the donor's semen — donors were accepted if the post-thaw analysis showed $\geqslant 20\times10^6$ motile spermatozoa/ml. Final acceptance also depended on the results of a number of pathology tests. Every semen sample from a donor was cultured in order to exclude significant bacterial contamination and if any potential pathogens were detected the sample was discarded. Since 1985 all donors have had the following blood tests: full blood examination (thalassaemia screen), blood typing (ABO and Rh), antibody to human immunodeficiency virus (HIV), antibody to cytomegalovirus (CMV), syphilis serology, and hepatitis B surface antigen. If a donor was positive for CMV antibody, then his semen was cultured for CMV and, if positive (4% of seropositive donors) his samples were not used for insemination. After 10–20 semen samples had been frozen, the stored semen was quarantined for a minimum of 6 months. The donor was then recalled and his blood re-tested for antibodies to HIV, syphilis, CMV and for hepatitis B surface antigen. If a donor seroconverted for any of these tests, his semen was not used in the programme. More recently, we have incorporated screening for hepatitis C, HIV-2 and human T-lymphotrophic virus strain one (HTLV-1).

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Cryoprotectant preparation

The glycerol–egg yolk–citrate (GEYC) cryoprotectant was prepared and stored as previously described (Matheson *et al.*, 1969), except that 0.1 mg/ml of kannamycin (Kannasig; Sigma Chemical Company, St Louis, USA), was used as antibiotic. Another modification was to adjust the glycerol concentration to 30% immediately before use so that the GEYC could be added to the semen in a 1:3 ratio to maintain a higher sperm concentration in the cryopreserved semen. This cryoprotectant gave minimal dilution of the semen and allowed a mean post-thaw motility recovery of 64%.

Semen cryopreservation

All semen samples were produced by masturbation in a room near the laboratory. Donors were instructed to have 3–5 days sexual abstinence before each appointment. The semen samples were then incubated at 37°C for 10–20 min to liquefy. Aliquots were taken for microbial culture within 15 min after ejaculation. After liquefaction, a drop was taken for microscopic examination prior to cryopreservation. One volume of cryoprotectant mixture was added to three volumes of semen. The cryoprotectant was added in approximately five aliquots over 10–15 min at room temperature. The resulting mixture was then aspirated into 0.5 ml French straws (B.I.C.E.F., 61302 L'Aigle Cedex, France), which were powder-sealed and individually labelled with the donor code and specimen number. The packaged semen was then cooled at 6°C/min from room temperature to –80°C and then plunged into liquid nitrogen (–196°C) for storage. When required, the straws were removed from liquid nitrogen and thawed in air at room temperature. The semen was warmed to 37°C prior to either post-thaw analysis or artificial insemination.

Donor insemination

Patients referred for DI had patent Fallopian tubes (laparoscopy/dye-hydrotubation; or hysterosalpingogram) and partners who were azoospermic (67%) or extremely oligozoospermic ($<2\times10^6$ spermatozoa/ml). Occasionally (<1%) patients were referred for genetic reasons.

Patients in the DI programme were monitored by daily measurement of luteinizing hormone (LH) and progesterone concentrations. When the morning LH showed a significant rise (>17 IU/l), insemination was performed that afternoon and the next morning. Patients known to be anovulatory were treated with clomiphene citrate. In about half of these patients ovulation was timed by a single dose of 5000 IU of human chorionic gonadotrophin (HCG) followed by a single insemination 42 h later if pre-HCG LH concentrations were still at baseline. All inseminations were high intracervical using 0.5 ml unwashed, cryopreserved donor semen. After 6–8 failed DI cycles patients were offered IVF using donor spermatozoa.

In-vitro fertilization (IVF)

Oocytes were retrieved after a routine human menopausal gonadotrophin (HMG)/clomiphene citrate stimulation protocol and inseminated in human tubal fluid medium (HTF; Irvine Scientific, Santa Ana, CA, USA) containing 10% human serum. Embryo culture was performed in HTF containing 1–10% human serum. The donor spermatozoa were routinely prepared by swim-up from a washed pellet or by separation on a mini-Percoll density gradient. Embryo transfers were performed at the 2–4-cell stage or cryopreserved (propanediol/slow freeze method) for later transfer. Establishment of a pregnancy was determined by β-HCG assay and fetal heart monitoring.

Statistical analysis

The data were analysed using maximum likelihood multivariate logistic regression analysis.

Results

Donor insemination using cryopreserved semen commenced at this Hospital in 1976. Between 1976 and 1991 a total of 5953 treatment cycles were recorded for a total of 816 pregnancies (13.7% fecundity). In order to investigate some of the donor factors determining success in DI/IVF, a more detailed analysis of 1986–1991 data was performed. The mean age for the IVF patients at the first treatment cycle was 32.9 ± 4.2 versus 31.2 ± 4.4 for the DI patients.

Analysis of donor IVF records for the study interval showed that spermatozoa from 56 donors were used in 303 IVF treatment cycles for 185 couples. There were 13 IVF cycles with complete fertilization failure (i.e. 4%) which were spread randomly among the donors. A total of 2421 oocytes were inseminated, of which 1573 (65%) underwent morphologically normal fertilization and cleavage. Subsequently, 634 embryos were transferred to the uterus during 298 cycles, resulting in elevated HCG (>250 IU/l) levels in 53 (18%) cycles, 37 successful pregnancies and 46 live deliveries (including seven pairs of twins and one triplet). There were also two ectopic pregnancies, two fetal deaths *in utero* (FDIU) and one stillborn baby. The other 11 were biochemical pregnancies or spontaneously miscarried. Transfer of cryopreserved embryos in 245 subsequent cycles resulted in a further 24 successful pregnancies and 29 live deliveries (including five pairs of twins). Thus, in total, 33% of couples had successful treatment with IVF using donor sperm. Transfer of cryopreserved embryos also resulted in one FDIU, two stillborn babies, four biochemical pregnancies and two spontaneous miscarriages.

The total fertilization rates for individual donors ranged from 12 to 82%. Logistic regression analysis using sperm variables, obtained at the initial semen test of each of 41 donors whose spermatozoa were used for both DI and IVF, showed that the primary correlate with fertilization rate in IVF was the pre-freeze motility (Figure 1a, $P < 0.001$, 28% variance explained). The other variables including sperm morphology, sperm count, post-thaw motility, percentage motility recovery and post-thaw motility after 3 h were not independently correlated with fertilization rate. There was no significant difference in fertilization or pregnancy rates between couples using fresh husband's spermatozoa versus frozen donor spermatozoa.

Analysis of DI results showed that during the 6 year interval, 44 new donors were used in 1915 treatment cycles for 640 patients resulting in 287 pregnancies (15% per cycle). This total includes 45 (15.7%) which subsequently miscarried, two (0.7%) stillborn and five (1.7%) ectopic pregnancies. The only abnormalities recorded were for a boy born at 28 weeks who was deaf, and another boy who was growth-retarded (1889 g). The successful pregnancies included 18 twins and two sets of triplets. The pregnancy rates per donor ranged from 0 to 41% per insemination cycle. Logistic regression analysis indicated that the primary variables correlated with donor pregnancy rates were the post-thaw motility (Figure 2b, $P < 0.001$), and the proportion of morphologically abnormal spermatozoa $P < 0.05$ (Figure 2b). The other variables examined were not independently correlated with pregnancy rates in donor insemination.

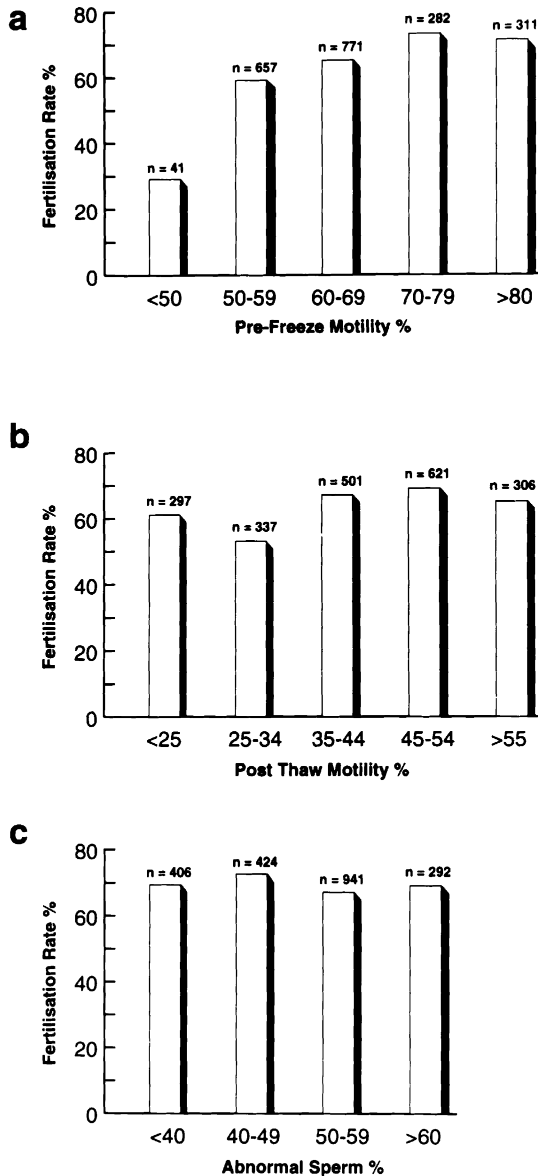

Figure 1. The relationship between the in-vitro fertilization normal fertilization rate (i.e. the total number of normally fertilized and cleaved oocytes as a percentage of the total number of oocytes inseminated, *n*), and (**a**) the pre-freeze motility (expressed as the percentage of progressing spermatozoa), (**b**) the post-thaw motility and (**c**) the sperm morphology (percentage abnormal).

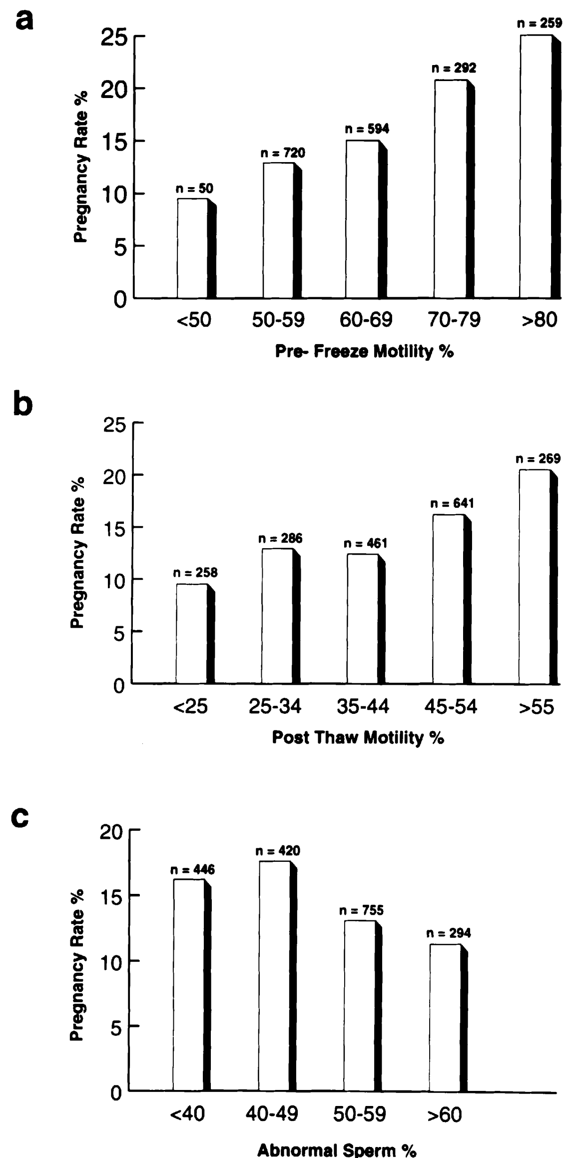

Figure 2. The relationship between the donor insemination pregnancy rate (fetal heart confirmed pregnancies/100 insemination cycles), and (**a**) the pre-freeze motility (percentage of progressing sperm), (**b**) the post-thaw motility and (**c**) sperm morphology (percentage abnormal).

Discussion

Efficient sperm banking was made possible by the inadvertent discovery that glycerol acted as an effective cryoprotectant (Polge *et al.*, 1949). The first report of the clinical use of spermatozoa frozen in the presence of glycerol appeared soon after this discovery (Bunge and Sherman, 1953; Bunge *et al.*, 1954). By 1956 this group had recorded nine pregnancies in 26 patients treated with cryopreserved spermatozoa (Keettel *et al.*, 1956). During the next 15 years the cryopreservation procedures were improved and higher pregnancy rates were reported. In 1973, Steinberger and Smith reported that cryopreserved semen was almost as effective as fresh semen when used clinically (Steinberger and Smith, 1973). In the same year Sherman reported the results of a comprehensive questionnaire and literature survey which allayed any concerns regarding the possibility of birth abnormalities resulting from the use of cryopreserved semen (Sherman, 1973). A total of 571 births were recorded, of which seven (1.2%) were abnormal and 50 (8.1%) conceptions resulted in spontaneous abortions. These rates were significantly lower than those recorded for the general population. These reports heralded the era of widespread sperm banking, including the establishment of many commercial sperm banks.

In 1977 Stanley Friedman reported his results from the treatment of 227 patients using cryopreserved donor semen

only (Friedman, 1977). His patients were treated for a total of 947 cycles, resulting in 91 pregnancies (9.6% per cycle). In 1980, David *et al.* reported a fecundity rate of 9.8% per cycle from treatment of 1188 patients between 1973 and 1978 (David *et al.*, 1980). They also found that the spontaneous abortion rate and the sex ratio were similar to those for natural reproduction. Of 529 women who conceived, four had ectopic pregnancies and one had a legal abortion. They did not mention any abnormalities at birth.

Our own results (13.7% for 1976–1991, 15% for 1986–1991) compare favourably with these larger studies, and with fecundity rates reported for the use of fresh semen of 10–20%. It has been estimated that the fecundity rate for natural conceptions is ~20–25%. For comparison, our highest yearly fecundity rate was 19.1% in 1981 from a total of 467 treatment cycles. In addition, insemination of donor semen with >80% pre-freeze motility resulted in 25% fecundity (Figure 2a). This suggests that under ideal conditions the conception rate for artificial insemination can approach that of natural reproduction.

Statistical analysis of our DI results using logistic regression indicated that donor pregnancy rates were significantly correlated with both the post-thaw motility and the sperm morphology. Thus, from Figure 2b it is apparent that the pregnancy rate doubles as the post-thaw motility increases from <25 to >55%. In contrast, there is a marked negative relationship between sperm morphology (% abnormal) and pregnancy rate (Figure 2c). These findings agree closely with those of David *et al.* (1980), Brown *et al.* (1988) and Le Lannou *et al.* (1995), but disagree with several other investigations which found no significant relationship between motility and pregnancy rates (Mahadevan *et al.*, 1982; McGowan *et al.*, 1983; Macleod and Irvine, 1995). There is no obvious reason for the different findings. They may be related to differences in motility assessment, donor or patient selection, cryopreservation technique, or statistical analysis of data.

Our IVF results indicate that normal fertilization rates are not compromised by the use of cryopreserved spermatozoa. The lack of correlation between fertilization rate and post-thaw motility may be due to compensatory effects of post-thaw sperm processing such as swim-up and subsequent adjustment of the motile sperm numbers added to each oocyte. Le Lannou *et al.* (1995) also found no significant relationship between the number of motile spermatozoa and IVF success, as measured by pregnancy rate.

By 1986, Sherman could report that there had been at least 24 000 births resulting from the use of cryopreserved donor semen, with abnormalities at birth of ~1% and 13% spontaneous abortion rate (Sherman, 1986). The sex ratio at birth was 51% male:49% female. In 1990 this figure was updated to 40 000 births, so that we can conservatively estimate that well over 50 000 births have occurred to date (Sherman 1990).

Since 1992 the number of patients requesting DI or donor IVF has decreased significantly because men with severe oligozoospermia can achieve pregnancies through intracytoplasmic sperm injection (ICSI). In addition, many men with obstructive azoospermia have had success using micro-epididymal or testicular sperm aspiration and subsequent ICSI. DI is now used mainly to treat patients with azoospermia due to totally absent spermatogenesis or patients with various genetic traits such as cystic fibrosis or thalassaemia. However, we anticipate that some patients who do not achieve a pregnancy with ICSI, or cannot afford it, will continue to request DI.

In conclusion, the results of this study have several implications for improvement of success rates in DI/IVF using donor spermatozoa: (i) selection of donors with good (e.g. ⩾60%) initial motility and with <60% morphologically abnormal spermatozoa is recommended; (ii) in order to achieve good pregnancy rates in DI (>10% per cycle) it is necessary to select donors with a post-thaw motility of ⩾25% and calculated motile sperm dose of 10×10^6/0.5 ml straw (i.e. 20×10^6/ml); it is important to use optimal sperm cryopreservation protocols so that, within the constraints outlined above, the highest proportion of potential donors can be accepted.

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