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SHORT COMMUNICATION

Repeated sperm injection under the zona following initial fertilization failure

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When fertilization fails following micromanipulative under-zona insemination, it is possible to repeat the procedure adding more spermatozoa to achieve fertilization, embryonic development and pregnancy. We report on 18 human in-vitro fertilization cycles where this approach was used. In nine cycles only late-fertilized embryos were available for transfer, and these gave rise to two viable pregnancies (22.2% per transfer). In six cycles, where a mixture of late- and timely fertilized embryos were available for transfer, two viable pregnancies arose (33.3% per transfer). In three cycles no fertilization was achieved even after reinsemination by repeated under-zona insemination.

Key words: in-vitro fertilization/micromanipulation/reinsemination/zona pellucida

Introduction

Currently the most commonly used micromanipulation technique for improved fertilization is the placement of spermatozoa under the zona pellucida. Since the first report of this technique (Ng *et al.*, 1988), several different centres have reported varying degrees of success (Cohen *et al.*, 1991; Fishel *et al.*, 1992; Sakkas *et al.*, 1992; Krzyminska *et al.*, 1992; Palermo *et al.*, 1992a). Following extensive experience with the use of partial zona dissection (PZD; Tucker *et al.*, 1991a), we have recently turned to the technique of under-zona insemination (SUZI) for treatment of both male factor and fertilization-failure infertility (Tucker *et al.*, 1993). In the event that SUZI is unsuccessful, the more invasive procedure of intra-cytoplasmic single spermatozoon injection may be adopted (Palermo *et al.* 1992b). We propose however, that even within the same in-vitro fertilization (IVF) cycle, any oocytes remaining unfertilized after initial SUZI may be reinseminated by SUZI using more spermatozoa. Not only does this approach allow rescue in certain instances of an otherwise failed IVF cycle, but it also allows assessment of spermatozoal numbers necessary to achieve fertilization with SUZI for future reference.

Materials and methods

Of the 18 couples considered here, six had experienced at least one failed IVF attempt due to idiopathic fertilization failure; the other 12 suffered from severe male factor infertility, and two of these had previously experienced fertilization failure following PZD. Average seminal quality (±SD) for all couples was: motility 35 ± 19%; count 14 ± 21 × 10^6/ml; strict morphology 13 ± 15% normal forms. The large standard deviations indicate the extremely poor quality of some of the specimens.

Our IVF techniques have previously been described (Tucker *et al.*, 1991b). Briefly, female partners underwent hypothalamic down-regulation with gonadotrophin-releasing hormone analogue, prior to ovarian stimulation with pure follicle stimulating hormone and human menopausal gonadotrophin. An average of 8.5 oocytes were collected per IVF cycle, and placed in droplets of culture medium under mineral oil. All oocytes were stripped of mature cumulus immediately following collection, scored for maturity, preincubated for 2–5 h in Earle's balanced salt solution (EBSS) containing 8% serum, and then exposed to 0.1% hyaluronidase before micromanipulation. All semen samples in this group of patients were preincubated for 15–20 min in 2.5 mmol/l pentoxifylline and 1 mmol/l 2′-deoxyadenosine, prior to processing by variants of the 'mini-Percoll' (Ord *et al.*, 1990) method. Between two to 12 spermatozoa were used initially for SUZI, subsequently adding up to 20 extra spermatozoa per oocyte at 10–18 h following initial insemination when no fertilization was observed. Micromanipulation of gametes was performed on a depression-well glass slide in a 5 μl droplet of culture medium under mineral oil, using Nomarski interference contrast optics. Although first-day SUZI was performed in a 0.1 mol/l sucrose solution, this was not found to be necessary for the repeat SUZI procedure, as sufficient perivitelline space was usually available for needle entry at this time. Sufficient spermatozoa were preloaded into a hollow glass needle with a 3–4 μm internal diameter at the bevel. Spermatozoa for the second-day reinsemination were always taken from the original stock of washed spermatozoa, which had been stored overnight at room temperature. Following reinsemination all oocytes and any embryos that arose were cultured in 15% maternal serum in EBSS until 3 days after oocyte collection.

All cleaving embryos for transfer underwent zona drilling with acidic Tyrode's to assist subsequent blastocyst hatching. The holes in the zona pellucida from the repeated SUZI procedures can cause multiple hatching sites, so compromising the implantation potential of the blastocysts. Such embryos may be rescued by

Table I. Breakdown of data from 18 cycles in 18 patients in which oocytes underwent repeated under-zona insemination (UZI)

	No transfer	Embryos from repeat UZI only	Mixed embryos from first day and repeat UZI
No. of patient cycles	3[a]	9	6
Monospermic fertilization rate	0/23	21/82	11/48 + 9/37[b]
Polyspermic fertilization rate	2/23	9/82	0/48 0/37
Cleavage failure rate	–	3/21	0/11 0/9
No. of embryo transfers	–	9 (18)[c]	6(20)
No. of viable pregnancies	–	2 (22.2%)	2 (33.3%)
No. of embryos implanting	–	2 (11.1%)	2 (10.0%)

[a]Three cycles with no monospermic fertilization following either UZI on first day or repeated UZI.
[b]Eleven embryos from first-day UZI, plus nine more embryos from repeated UZI on the remaining 37 unfertilized oocytes.
[c]Number of transferred embryos in brackets.

drilling a larger hole before uterine transfer (Cohen and Feldberg, 1991).

Results

Table I shows the 18 cycles (18 patients) in which repeat SUZI was performed in an attempt to achieve fertilization after initial failure of SUZI on the day of oocyte collection. In three cycles no fertilization was achieved either initially or following addition of extra spermatozoa under the zona pellucida on the day after oocyte collection. In six cycles 11 monospermic zygotes arose from first-day SUZI following micromanipulation of a total of 48 oocytes (22.9% fertilization rate), then a further nine monospermic zygotes were generated by repeat SUZI; thus a total of 20 embryos was produced for transfer, of which two implanted (viable pregnancy rate per transfer, 33.3%; embryonic implantation rate of 2/20, 10%). More interestingly, in nine cycles no fertilization occurred in 82 oocytes following first-day SUZI. After repeating the SUZI with more spermatozoa, 21 monospermic zygotes (25.6% fertilization rate), and nine polyspermic zygotes (11.0% of oocytes) arose. Three monospermic embryos failed to cleave (14.3% of zygotes), while 18 were transferred in nine women, giving rise to two singleton pregnancies (viable pregnancy rate per transfer, 22.2%; embryonic implantation rate, 11.1%).

The average number of spermatozoa injected initially per oocyte was 7.0 ± 2.7, and after fertilization failure an additional number averaging 13.6 ± 4.5 spermatozoa was added giving a total average number of spermatozoa placed under the zona pellucida of 20.6 per oocyte. The average cell number of the 11 embryos produced from first-day SUZI was 6.7 ± 2.8 blastomeres per embryo, compared to an average cell number of 4.8 ± 1.9 blastomeres per embryo in the 18 embryos produced from repeat SUZI. The total fertilization failure from SUZI performed on the day of oocyte collection in 12 cycles included four couples who had suffered previous IVF failure, and eight couples with severe male factor infertility. Consequently, little of significance could be drawn from which seminal factors may have caused this initial fertilization failure in such a small group of patients. Furthermore, the three cycles in which no monospermic fertilization was achieved even after repeated SUZI included one couple with a normozoospermic sample.

Discussion

It has been established that embryos generated following reinsemination have an intrinsically poor implantation potential. Even if their delayed development is compensated for by freezing and adjusted thawing in subsequent cycles (Tucker *et al.*, 1991b), embryonic implantation rates still remain as low as 3.3% per embryo.

In the present report, however, embryos generated by repeat SUZI reinsemination had an implantation potential of >10% per embryo, even though their development was marginally slowed by late fertilization. From this it can be inferred that in the general non-male factor infertile population, fertilization failure is a function of poor oocyte quality, and consequently any embryos that arise following reinsemination are probably of poor quality. Conversely, in male factor patients or those suffering from idiopathic fertilization failure, it is possible to generate apparently good quality late-fertilized embryos following reinsemination, in this instance by repeated under-zona insemination. This implies that intrinsic gamete quality is not at fault in the initial failure to fertilize, but that gametic fusogenic potential alone was poor. Having overcome this problem, chances of normal embryonic development seem comparable with that in standard IVF procedures.

In the event that fertilization fails following SUZI on the day of oocyte collection, it is conceivable that recourse to the more invasive procedure of direct ooplasmic injection is possible to attempt to rescue what would otherwise be a failed IVF cycle. Oocyte damage or poor embryonic development as a result of this course of action in our clinic has led us to favour repeated SUZI. There may exist a distinct learning curve for successful application of direct ooplasmic injection; nevertheless, the results of Palermo *et al.* (1992b) indicate that cleavage failure may be high and poor embryonic development common, following direct intra-cytoplasmic injection (if all 60 cases in their study are considered).

It is arguable that we should initially place more spermatozoa under the zona to obviate the need for repeated micromanipulation to achieve late fertilization, although this may increase our overall polyspermy rate. For example, Krzyminska *et al.* (1992) chose to place greater initial numbers of spermatozoa under the zona (approximate average of 17.5 sperm/oocyte), whereas Fishel *et al.* (1992) chose to inseminate lower numbers (average 4.5

sperm/oocyte); their differing policies were clearly reflected in the respective monospermic and polyspermic fertilization rates. Our policy lies somewhere between these two reports, initially inseminating on average 7.0 sperm/oocyte in this group of patients. Currently no one factor or group of factors has been defined as having a direct bearing on the number of spermatozoa necessary for insemination under the zona pellucida, in order to achieve consistent monospermic fertilization. Clinical assessment on a case-by-case basis to evaluate fluctuating gamete quality may be helpful, and previous experience from SUZI attempts will also aid in estimating numbers of spermatozoa to be inseminated. In the present short report fertilization failure following SUZI was seemingly unrelated to sperm quality. The standard semen analysis proved of little value for predicting outcome in a large series of microsurgical attempts (Cohen *et al.*, 1992a), and this strongly suggests that we should be studying more carefully the surface molecular components central to gamete fusion, to improve our definition of fertile gametes (Tucker and Chan, 1993).

In the meantime, although techniques to correct polyspermy are under development (Malter and Cohen, 1989), it appears that it is preferable to choose lower numbers of spermatozoa for initial SUZI to avoid excessive polyspermy. In the event that initial fertilization is poor, it is possible to follow up with a repeated attempt at SUZI. In the present report all SUZI embryos underwent assisted hatching (Cohen *et al.*, 1992b) prior to uterine transfer. This means that those embryos that arose from repeated SUZI underwent three micromanipulative procedures and still retained viability in 11% of cases.

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