CLINICAL ASSISTED REPRODUCTION

Pregnancy Rates After Intracytoplasmic Sperm Injection in Relation to Sperm Recovery Techniques

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Purpose/Methods: Clinical outcome after intracytoplasmic sperm injection (ICSI) was evaluated in relation to three techniques of sperm recovery, mini-Percoll, simple concentration, and centrifugation and washing.

Results: Whereas fertilization and embryonic cleavage rates were similar in the three groups, the rates of implantation and clinical pregnancy were statistically significantly higher following sperm recovery by the techniques of mini-Percoll and centrifugation and washing.

KEY WORDS: pregnancy rates; intracytoplasmic sperm injection; sperm recovery techniques.

aspirated directly from the testicular tissue have been utilized for ICSI achieving fertilization, good-quality embryos with successful implantation, clinical pregnancies, and live births (4). These observations led us to question the relevance of applying the various sperm preparation techniques employed in conventional in vitro fertilization to intracytoplasmic sperm injection. This study examines the fertilization, clinical pregnancy, and implantation rates after ICSI in relation to three methods of semen preparation utilized in our unit: (i) mini-Percoll, (ii) simple concentration, and (iii) centrifugation and washing.

INTRODUCTION

To be potentially functional, ejaculated spermatozoa must be separated from seminal plasma quickly and efficiently. The success or applicability of a semen preparation technique is often assessed by its yield of motile and morphologically normal spermatozoa (1). This yield influences the fertilization rates, embryo quality, and pregnancy and implantation rates following conventional in vitro fertilization and embryo transfer (IVF-ET) procedures (2,3). The successful introduction of ICSI has made it possible to utilize sperms recovered from unorthodox sperm preparation techniques. For example, spermatozoa extracted or

MATERIALS AND METHODS

One hundred ten intracytoplasmic sperm injection-embryo transfer (ICSI-ET) treatment cycles performed on 95 couples with severe male-factor infertility (sperm density, <5 million/ml) or with previous failed fertilization from conventional IVF-ET at the IVF Centre, Maternity Hospital, Kuwait, from November 1994 to June 1996 were studied with respect to the techniques of sperm recovery from the ejaculate.

The evaluation of semen density and motility was carried out according to the recommendations of the World Health Organisation (5). Strict criteria were used to evaluate sperm morphology (6). Semen evaluation and preparation were done at least once prior to the treatment cycle. Ejaculated semen samples were reevaluated on the day of oocyte pickup and prepared according to the following criteria: (i) mini-Percoll if the sperm density is >1 million/ml, (ii) simple whole semen concentration, or (iii) centrifugation and wash-

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ing when only 1–2 sperms were seen per entire high-power field (<1 million/ml).

Throughout the study period, all semen samples with a sperm density >1 million/ml, which included all cases of previous failed fertilization from conventional IVF (65 treatment cycles), were prepared using the mini-Percoll discontinuous gradient technique adapted from methods described in detail previously (7). Commercially prepared 100% isotonic Percoll (Medi-Cult, Copenhagen, Denmark) was diluted to 95, 70, and 50% isotonic gradients with Earle's balanced salt solution (EBSS), keeping the gradients isosmotic. The discontinuous density gradients were prepared by carefully layering 0.3 ml of 95, 70, and finally 50% Percoll solutions in a 5-ml conical tube starting with the highest-density solution at the bottom. One-half milliliter of semen was placed on the top of the gradient and the tube centrifuged for 25 min at 300g. Following centrifugation, the seminal fluid layer and the 50, 70, and most of the 95% Percoll solutions were removed. The soft pellet was washed out in two steps with EBSS and centrifuged twice for 10 min at 500g as recommended by Pickering and colleagues (8). The final 1 ml of sperm stock was analyzed and incubated until the time of ICSI. In 32 treatment cycles (November 1994 to October 1995), semen samples with 1–2 sperms per entire high-power field were prepared by simple whole semen concentration. The whole semen sample was spun at 600g for 15 min and the supernant was discarded (5). The unwashed pellet was analyzed and incubated until used for ICSI. From November 1995 until the end of the study period, semen samples with 1–2 sperms per entire high-power field (13 treatment cycles) were prepared by the technique of centrifugation and washing. The semen sample was mixed in multiple tubes 1:10 with medium, then centrifuged at 200g for 5 min, and the supernants were discarded. The pellets were pooled together, suspended in fresh medium, and centrifuged a second time to concentrate the sperms. The final sperm stock was incubated until the time of ICSI.

All female partners underwent controlled ovarian hyperstimulation. The details of the stimulation and oocyte retrieval have been described elsewhere (9). The cells of the cumulus and corona radiata were removed by incubation for 30 sec in commercially prepared Earle's medium with 80 IU hyaluronidase/ml (Medi-Cult, Copenhagen, Denmark) under paraffin. The removal of the cumulus and corona cells was enhanced by aspiration of the complexes in and out of hand-drawn glass pipettes. Afterward, the oocytes were rinsed several times in microdroplets of EBSS

under paraffin. The oocytes were examined under the microscope at 200× magnification, to assess the presence or absence of a germinal vesicle or the first polar body. The oocytes were then incubated in microdroplets of EBSS under paraffin in an atmosphere of 5% CO₂. Examination was performed again after 4–6 hr to see whether more oocytes had extruded the first polar body. ICSI was performed on morphologically intact oocytes that had extruded the first polar body (metaphase 2 oocytes). The technique of ICSI employed was adapted from methods described previously (10,11).

Research Instruments (United Kingdom) micromanipulator equipment and commercially prepared holding and injection pipettes (Research Medical Laboratory, Australia) were used. One microliter of prepared sperm stock was added to 2–4 µl of commercially prepared polyvinylpyrrolidone (PVP; Medi-Cult, Copenhagen, Denmark). The 3–5 sperm–PVP droplet was placed in the center of a petri dish (Falcon Type 1006) and surrounded by 2 microdrops of EBSS supplemented with 10% human serum albumin. Morphologically normal-looking sperm was immobilized by stroking the tail and was aspirated tail-first into the tip of the injection pipette. Sperms prepared by simple concentration were further “cleaned” by aspirating the sperm three to five times in and out of the injection pipette in a separate PVP droplet in the petri dish. The oocyte was held by slight negative pressure exerted on the holding pipette with the polar body at 12 or 6 o'clock. A single spermatozoon was injected into the ooplasm after a gentle aspiration of the ooplasm to ensure that the oolema was broken. Further culture of the oocyte and embryo handling was performed as described previously in detail (9). A maximum of the three best-quality embryos was transferred in patients less than 35 years old, and a maximum of four in older patients, transcervically to the uterine cavity about 48 hr after ICSI. An analysis of 19 treatment cycles of ICSI-ET with spermatozoa extracted or aspirated from the testicular tissue or the epididymis also performed during this study period is the subject of a separate report.

Statistical Analysis

The Mann Whitney test was used to determine whether the differences observed between the three groups separately were statistically significant ($P < 0.05$).

RESULTS

The mean age of the female partners and laboratory and clinical outcome statistics are summarized in Table I. Of 1416 oocytes retrieved at metaphase 2 and of good quality, 1043 (74%) were injected. The fertilization rate as evidenced by the presence of two pronuclei was lowest (61%) when sperms were recovered by the centrifugation and washing technique (group 3), the difference ($P > 0.05$) not being statistically significant with group 1 or 2. Fertilization rates in groups 1 and 2 were similar (75 vs 77%). The overall fertilization rate was 74%, with 10% of the injected oocytes being damaged during the procedure. The cleavage rates were similar in the three groups (86, 84, 86%). The mean number and grade of embryos transferred were also similar. The clinical pregnancy (embryonic heart beat seen) rate per transfer was lowest in group 2 (sperms retrieved by simple concentration). Compared with group 3 (22 vs 54%), the difference was statistically significant ($P = 0.039$). The difference in clinical pregnancy rates in groups 2 and 1 (22 vs 41.5%) was not statistically significant ($P = 0.077$). Clinical pregnancy rates in groups 1 and 3 were not statistically significantly different (41.5 vs 54%; $P = 0.359$). The implantation rate (heartbeat per embryo transfer) was lowest in group 2 (9.3%). The higher implantation rates in groups 1 (23%) and 3 (19%) were statistically significantly different when each was separately compared with group 2 ($P = 0.041$ and 0.045, respec-

tively), while no statistical significant difference was observed between group 1 and group 3 ($P = 0.713$).

DISCUSSION

In this observational study, the three methods of sperm recovery for ICSI evaluated did not appear to be associated with differing rates of fertilization and embryonic development. In contrast, whereas the clinical pregnancy and implantation rates were similar for groups 1 and 3, a statistically significant decreased rate was seen following sperm recovered by the simple concentration technique (group 2). Our findings following ICSI using sperm recovered from semen prepared by the mini-Percoll technique are in accord with the literature (12). The mechanism by which Percoll acts as a selecting and possibly protective medium is not yet fully understood. Van Der Zwalmen and colleagues (13) have suggested a possible reduction of membrane dysfunction due to excessive production of reactive oxygen species or an elimination of factors which may interfere with one of the steps of fertilization or simply a selection of a superior sperm fraction. However, sperm may not be recovered when an ejaculated semen sample with 1 or 2 sperms per the entire high-power field (<1 million/ml) is subjected to mini-Percoll. The simple concentration or centrifugation and washing techniques provided a sufficient number of sperm for use in ICSI in these cases. Although the

Table I. Clinical and Laboratory Details of the Three Groups in Relation to Treatment Outcome

	Group 1 (<i>n</i> = 65), mini-Percoll (%)	Group 2 (<i>n</i> = 32), simple conc (%)	Group 3 (<i>n</i> = 13), centrif./wash (%)	Total (<i>n</i> = 100) (%)
Mean age (+ 1 SD)	32.5 ± 4.9	31.8 ± 5.1	32.3 ± 4.3	
Number of oocytes collected	811	433	172	1416
Number of oocytes at metaphase 2 stage	616 (76)	323 (75)	149 (87)	1088 (77)
Number of oocytes injected	579 (71)	322 (74)	142 (83)	1043 (74)
Number of oocytes with 2 pronuclei	436 (75)	247 (77)	87 (61)	770 (74)
Number of oocytes damaged	67 (12)	32 (10)	9 (6)	108 (10)
Number of oocytes cleaved	375 (86)	207 (84)	75 (86)	657 (85)
Number of embryos transferred	210	107	48	365
Mean number of embryos transferred	3.2 ± 0.03	3.3 ± 1.0	3.7 ± 0.48	
Mean grade of embryo	2.8 ± 0.3	2.8 ± 0.3	3.0 ± 0	
Pregnancy rate per embryo transfer	27 (41.5)	7 (22)	7 (54)	41 (37.3)
Implantation rate	48 (23)	10 (9.3)	9 (19)	67 (18.4)

simple concentration procedure did not appear to be associated with diminished sperm function at the embryological level (fertilization, cleavage, and morphological embryo quality) as assessed in the laboratory, a clear decrease in implantation rate was seen following sperm prepared in this way. The possible explanation is that, unlike the mini-Percoll or centrifugation and washing techniques, the sperm recovered by simple whole semen concentration was not washed to remove the seminal plasma. The many compounds in seminal plasma adherent to the sperm at ICSI could impair the embryonic potential to implant. In contrast, the findings were similar when sperm from either the mini-Percoll or the centrifugation and washing technique was used for ICSI. The need to separate rapidly and effectively sperm from the seminal plasma is a routine step in IVF procedures. Our observations confirm that these techniques should also be routinely used when ICSI is indicated. The simple technique of centrifugation and washing appears to be as efficient as the mini-Percoll and should be used when the sperm density in an ejaculated semen sample is only one or two per entire high-power field.

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