

CUMULUS REMOVAL AND ADDITION OF FOLLICULAR FLUID POSSIBLY IMPROVES PREGNANCY RATES WITH IN VITRO FERTILIZATION FOR MALE FACTOR

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This study evaluated the use of in vitro fertilization (IVF) for patients with subnormal semen parameters without the use of micromanipulation. All patients were characterized as having male factor as follows: normal morphology (NM) $\leq 10\%$ according to strict criteria [15] and motile density (MD) $\leq 10 \times 10^6/\text{mL}$. Strict morphology was divided into three groups: group I ($n = 72$), $\leq 2\%$ group II ($n = 24$), 3–5%; and group III ($n = 29$), 6–10%. Modification of standard IVF techniques included manual cumulus removal (CR) from oocytes, pooling up to ten oocytes together in 1 mL of media, and supplementing media with 20% human follicular fluid (FF). Rates of fertilization and pregnancy were compared. The overall fertilization rate (FR) was 57.7% and the pregnancy rate (PR) per retrieval cycle was 14.8%. There was no significant improvement in the fertilization or PRs when IVF was modified using CR and FF, although the FR was higher in group I for patients who received the modified procedures. In patients with $\leq 5 \times 10^6$ sperm/mL, there were no pregnancies in five cycles and four transfers following the conventional method, but two sets of twins with the modified protocols in seven cycles. Clinical pregnancies were achieved with male factor without the need for micromanipulation. The most severe cases were automatically assigned to modified IVF techniques, e.g., CR with or without FF. Prospective randomized studies are needed to determine if modified procedures are superior to conventional therapy.

Keywords micromanipulation, oligoasthenozoospermia, teratozoospermia, oocyte pooling

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One of the indications for in vitro fertilization-embryo transfer (IVF-ET) is male factor. However, a problem in assessing the efficacy of IVF for male factor lies in identifying the subfertile male based on merely the semen analysis [7, 8]. Some semen samples with subnormal count, motility, and/or morphology are capable of fertilizing oocytes in vitro [11], while sperm that appears normal may actually be incapable of resulting in pregnancy [6]. Once the problem has been identified as male factor, the difficulty is in selecting an appropriate treatment. When conventional techniques are not effective in achieving fertilization, newer procedures, e.g., micromanipulation and modified insemination techniques, are available [12, 13, 17, 20, 22]. Unfortunately, current treatment methods vary in the degree of reported success, potential damage to gametes, and cost.

Cohen et al. [12], advocated micromanipulation for three different groups based on normal morphology, motility, and sperm concentration. One of these criteria involves poor strict morphology (SM) ($\leq 2\%$ normal) as suggested by Kruger et al. [15]. There are some data that state that pregnancy rates (PRs) following IVF-ET are quite poor with SM $< 4\%$ normal [16], but other data suggest that this test does not accurately distinguish the subnormal male population, even with an in vivo setting [8]. Other laboratories have reported success with male factor patients without the use of micromanipulation. Some of these programs utilize techniques that bring the gametes in closer proximity, such as combining the sperm and oocytes in microdroplets [21] or in capillary tubes [1, 14]. Other techniques, such as sperm pretreatment with follicular fluid (FF) [13] or pentoxifylline [20] may enhance sperm function.

The study presented herein retrospectively evaluated the efficacy of nonmicromanipulation for male factor, including two modified in vitro insemination techniques, i.e., removal of the cumulus cells with or without the addition of FF, in couples with "male factor" problems undergoing IVF-ET.

MATERIALS AND METHODS

Patient Selection. A total of 81 consecutive IVF cycles between 1 September 1991 and 2 February 1993 in which the male partner was diagnosed as severe male factor were included in this study. In a pre-IVF semen analysis, all patients were found to have $\leq 10\%$ normal morphology (NM) according to strict criteria [15] and motile density (MD) $\leq 10 \times 10^6/\text{mL}$ in the initial semen sample on the day of IVF.

In Vitro Fertilization. One group ($n = 36$) received the conventional insemination (protocol A): one to four oocytes cultured per 1 mL of human tubal fluid (HTF) (Irvine Scientific, Santa Ana, CA, USA) with 0.5% bovine serum albumin (BSA). Another group ($n = 32$) received the cumulus removal (CR) (protocol B): Cumulus oophorus was manually dissected away from oocytes and 1–10 oocytes were cultured per milliliter HTF. The third group ($n = 13$) was treated with (protocol C): The same procedure was followed as in protocol B, but HTF was supplemented with 20% FF. Protocol A was used on all patients unless the MD was so poor that the minimum of 10,000 motile sperm with NM per oocyte was not available, or, if there was an adequate number of sperm, but poor progression. Only patients in which the female partner did not have serum antisperm antibodies (ASA) were included in group C.

In all three groups, oocytes were cultured in organ culture dishes (Falcon 3037) in a humidified incubator at 37°C with $5\% \text{CO}_2$ in air. Culture medium was overlaid with mineral oil (Squibb). The FF used in this study was pooled from two or more mature follicles during the patient's retrieval, then centrifuged at $795g$ (Clay-Adams Dynac II tabletop centrifuge) for 10 min. Oocytes in all three groups were inseminated with $\leq 10 \times 10^3$ motile sperm with normal strict morphology per oocyte. Otherwise,

the total washed sperm was divided evenly between insemination dishes. Sixteen hours after insemination, corona cells were removed using gentle suction through a fine-bored Pasteur pipet and checked for the presence of pronuclei and polar bodies. Oocytes containing 2 pronuclei (2PN) were considered fertilized and those with a visible polar body were considered mature.

Sperm Preparation. Sperm was processed using Percoll density gradient. Percoll columns were prepared by layering 1 mL each of 90, 60, and 45% Percoll made isotonic using modified HTF (modified HTF-Hepes 10 \times , Irvine Scientific #90141). Semen was diluted 1:1 with HTF, then up to 2 mL of the mixture was overlaid onto the prepared columns and centrifuged for 20 min at 300g. The top semen layer was discarded and the sperm pellet was placed into a new tube. The pellet was washed twice and then resuspended in up to 0.5 mL of HTF (Irvine Scientific). A modified procedure in which patients ejaculate into 5-mL Earle's balanced salts solution (EBSS) containing 0.1 M galactose and 5 mg chymotrypsin was used for patients with ASA in their semen or if the sample was highly viscous [2]. For some patients with low MD, two samples were collected and combined after processing.

The PR and fertilization rate (FR) for mature oocytes were evaluated according to the insemination protocol A, B, or C. Strict morphology was divided into three groups: group I ($n = 27$), $\leq 2\%$; group II ($n = 24$), 3–5%; and group III ($n = 30$), 6–10%. Any pregnancy from subsequent frozen ET were included in the PRs.

RESULTS

Fertilization rates and PRs using protocols A, B, and C in groups I, II, and III are shown in Tables 1, 2, and 3. The FR for patients in the combined groups was 57.7%. The PR per retrieval cycle was 14.8% for the combined groups and 18.2% when cycles without fertilization were excluded. While the FR seemed to improve when protocols B and C were used in group I, the difference was not significant (Table 1). In all three groups the sample sizes were too small to make any inferences (Tables 1, 2, and 3). In patients with $\leq 5 \times 10^6$ sperm/mL, there were no pregnancies in five cycles and four transfers following protocol A, but one set of twins each with protocol B and C in seven cycles.

DISCUSSION

It is not yet clear what factors should be included in the definition of severe male factor. While various semen parameters can indicate a reduced chance of success with fertilization,

TABLE 1 IVF Pregnancy Rates Without Micromanipulation in Oligospermic Males With $\geq 2\%$ Normal Strict Morphology

Parameter	Protocol A	Protocol B	Protocol C
Number of patients	6	17	4
Number of cycles with fertilization	4 (66.7%)	14 (82.3%)	4 (100%)
Number of mature oocytes fertilized/ number of mature oocytes	22/64 (34.4%)	84/133 (63.1%)	36/59 (61.0%)
Number of clinical pregnancies	1	2	1
Pregnancy rate per cycle	16.7%	11.8%	25.0%
Pregnancy rate per cycle with fertilization	25.0%	14.3%	25.0%

TABLE 2 IVF Pregnancy Rates Without Micromanipulation in Oligospermic Males With Normal Morphology >2% and ≤5%

Parameter	Protocol A	Protocol B	Protocol C
Number of patients	14	6	4
Number of cycles with fertilization	10 (71.4%)	4 (66.7%)	2 (50%)
Number of mature oocytes fertilized/ number of mature oocytes	55/91 (60.4%)	30/56 (53.6%)	6/20 (30.0%)
Number of clinical pregnancies	2	0	0
Pregnancy rate per cycle	14.3%	0.0%	0.0%
Pregnancy rate per cycle with fertilization	20.0%	0.0%	0.0%

they cannot accurately predict the outcome following IVF [5]. One of the goals of this study was to analyze the success of patients in our laboratory who have subnormal MD and subnormal morphology using strict criteria. Our results confirmed that subnormal semen parameters cannot accurately predict subsequent poor outcome, since average fertilization and PRs were demonstrated following IVF-ET for male factor patients. Perhaps other factors such as hypo-osmotic swelling (HOS) scores or previous failure to fertilize any oocytes may better define the male factor patient, but even these have been shown to be inconsistent [4, 9].

Appropriate treatment for male factor patients should be based on the severity of the problem, and high-cost treatments, such as micromanipulation, should be reserved for the most severe cases. In our program, we attempt to enhance the cell culture environment to improve the conditions necessary for IVF for male factor patients. This is done by removing the cumulus cells from around the oocytes, pooling more oocytes together in a small volume, and adding FF to the culture medium. The removal of cumulus cells from around the oocyte may improve the chances of sperm with low or abnormal motility to come in contact with the zona pellucida. Cumulus removal also allows pooling more oocytes together in the culture dishes to increase the proximity of sperm and oocytes. Human FF been found to contain factors that improve the rate of fertilization of human oocytes in vitro [13], possibly by enhancing sperm motility [3], or by providing factors necessary for the completion of the acrosome reaction in vitro [19, 18].

TABLE 3 IVF Pregnancy Rates Without Micromanipulation in Oligospermic Males With Normal Morphology >5% and ≤10%

Parameter	Protocol A	Protocol B	Protocol C
Number of patients	16	9	5
Number of cycles with fertilization	15 (93.7%)	8 (88.9%)	5 (100%)
Number of mature oocytes fertilized/ number of mature oocytes	96/127 (75.6%)	62/133 (46.6%)	47/76 (61.8%)
Number of clinical pregnancies	3	1	2
Pregnancy rate per cycle	18.75%	11.1%	40%
Pregnancy rate per cycle with fertilization	20.0%	12.5%	40.0%

The study presented herein was not designed to compare the efficacy of standard and modified techniques. We were able, however, to demonstrate a clinical PR in patients with male factor that compares favorably with PRs in non-male factor cases without the use of micromanipulation. In general, protocol A was used for better quality sperm and protocol C was used for the poorest. The highest PRs were seen following addition of FF (protocol C), at least suggesting that CR and FF may improve PRs. However, a prospective randomized study is needed to determine whether CR, adding FF, or pooling more oocytes improves the PRs and FRs compared to standard protocol.

Independently, Ord et al. [17] reported good PRs following IVF-ET for male factor without the need for micromanipulation using similar procedures compared to the study presented herein. Ord's technique differed from our method as follows: (1) The embryos are cultured in tubes rather than organ culture dishes; (2) human serum albumin (HSA) is the only protein source in the medium; and (3) semen is processed using three 0.3-mL Percoll layers with a longer (30–45 min) centrifugation time.

As in our procedure, Ord et al. incubate sperm and oocytes together in 1.0 mL of culture media as opposed to using a microdrop insemination, which is commonly 0.2 mL or less. The larger volume allows more oocytes to be pooled together with a greater number of sperm. Theoretically, microdrop inseminations risk the depletion of medium components and the accumulation of toxic metabolites from sperm and oocytes which may be diluted in a larger volume. Nevertheless, prospective randomized studies are needed to compare the efficacy of larger versus smaller insemination volumes.

The fact that males with subnormal MD and strict morphologic criteria ($\leq 2\%$) had results similar to males with higher levels suggests that "poor" morphology should not be an indication for micromanipulation. This is in contrast to the study published by Oehninger et al. [16]. Possibly new semen parameters will be defined that could identify male factor cases that require micromanipulation. Large IVF centers with established successful micromanipulation results could randomly assign couples requiring IVF to either micromanipulation or nonmicromanipulation enhancing techniques when the male is subnormal, as defined by WHO criteria, or when previous FRs using conventional techniques have been poor. This type of study should help to determine under what conditions it is cost effective to use micromanipulation.

Cases have been identified in which a couple with male factor achieves an acceptable FR yet another apparently normozoospermic couple failed or had poor fertilization with the same oocytes using a shared oocyte technique in which two couples equally share a harvested pool of oocytes [10]. In other cases, couples with failed fertilization, presumably due to male factor, have subsequently achieved fertilization when donor oocytes were used [11]; some of these cases may be related to zona pellucida abnormalities that can be overcome by micromanipulation. Furthermore, about one-third of couples with failed fertilization were successful in producing embryos in a subsequent cycle even using standard insemination procedures [11].

We have shown that pregnancies can be achieved without the use of micromanipulation in patients with male factor. Because micromanipulation procedures are often expensive, they should not be used indiscriminately. Furthermore, the implications of bypassing the natural sperm selection process and the risk of selecting abnormal sperm for micromanipulation have not been fully investigated. Only a minority of IVF centers have the means to perform micromanipulation. If nonmicromanipulation enhancing procedures are found to help a segment of

the infertile population, then assisted reproductive technology may become available to a larger group of patients who fail to benefit from standard techniques.

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