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The Limitations of in Vitro Fertilization from Males with Severe Oligospermia and Abnormal Sperm Morphology

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Thirty-one patients whose infertility was attributed to oligospermia were included for treatment by in vitro fertilization and embryo transfer. Three subgroups were defined: severe oligospermia (≤5 million motile sperm/ml), moderate oligospermia (6 to <12 million motile sperm/ml), and abnormal sperm morphology (>60% atypical). The fertilization rates were compared to those of a normospermic group managed concurrently. A modified overlay technique of sperm preparation is described for oligospermic samples so that the number of motile spermatozoa inseminated into each tube or culture dish containing a mature preovulatory oocyte was similar in each category, within the range 0.5 to 2 × 10^5/ml. Significantly fewer oocytes were fertilized in the severe oligospermic group ($P < 0.001$), suggesting a reduced capacity for fertilization by spermatozoa from severely oligospermic males. The fertilization rate of oocytes was normal in the moderate oligospermic group and those with abnormal morphology, although in the latter there was a significant delay noted in reaching the pronuclear stage ($P < 0.001$), and the embryos were at a less advanced stage of cleavage at the time of transfer ($0.001 < P < 0.01$). Pregnancies were achieved in both the severe and the moderate oligospermic groups, with healthy infants delivered from each.

KEY WORDS: in vitro fertilization (IVF); oligospermia; ova; spermatozoa; abnormal sperm morphology; infertility; pregnancy.

INTRODUCTION

In an historical study on a large series of fertile and infertile men, MacLeod and Gold demonstrated that more than 80% of fertile males have a sperm density greater than 40 million/ml (1). Further epidemiological studies have since been reported with some variation of opinion regarding the relevant lower limit for sperm density (2,3). However, a consistent correlation with infertility appears to exist only when the sperm concentration falls below 20 × 10^6 spermatozoa/ml and this is the level agreed on by the World Health Organization (WHO) task force on the diagnosis and treatment of infertility (4). In addition, the WHO Laboratory Manual describes the standard conditions for collection of the semen sample, its delivery, and the standardization of laboratory procedures indicating that "a normal sample will show 60% or greater motile spermatozoa with a majority exhibiting good to excellent forward progressive movement at one-half hour to three hours after ejaculation." Nonetheless, major problems do exist in ascertaining whether a particular male has fertile, subfertile, or infertile semen because of individual variability in both the total number of spermatozoa and the volume of the ejaculate (5) as well as the influence of other fertility parameters determining conception. Since the introduction of the zona-free hamster penetration test, several workers have compared the performance of spermatozoa from fertile and infertile males with a view to developing a precise in vitro assessment of the potential for spermatozoa to fertilize oocytes. Generally, the results have indicated reduced rates of binding and penetration of zona-free hamster ova (6) from infertile males.

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Most cases of oligospermia (or oligozoospermia) are of unknown cause but there are some well-known associations with chromosomal disorders, radiation, mumps orchitis, treatment with cytotoxic drugs, abnormalities of testicular descent, gonadotrophin deficiency, genital tract obstructions often arising from infection, and the presence of a varicocele (7). A range of empirical therapies is available for idiopathic chronic oligospermia but the mainstay of treatment for those failing to conceive is the use of donor spermatozoa. More recently the techniques of intracervical and intrauterine insemination of washed spermatozoa have been applied (8,9) and it has been suggested that in vitro fertilization should be assessed as a potential form of treatment for various nontubal causes of infertility, including oligospermia (10). Patients exhibiting prolonged periods of oligospermia who had failed to conceive by intrauterine insemination using husband's washed sperm were included in our in vitro fertilization and embryo transfer (IVF-ET) program as a combined therapeutic and diagnostic study to assess gamete compatibility.

MATERIALS AND METHODS

The IVF-ET program at PIVET Laboratory functions in collaboration with the University of Western Australia and has established a viable service program (11). It was initiated for the management of infertility due to irreparable tubal disease (12) and has now included some patients with other forms of infertility who have proven resistant to conventional treatments such as cases with endometriosis, cases with heterologous sperm antibodies (13), and this series of 31 couples whose infertility was attributed to chronic oligospermia or abnormal sperm morphology as the sole cause (21 cases) or a major coexistent factor with occlusive tubal disease (10 cases).

Subclassifications of oligospermia vary from those which simply consider density to those which attempt to take sperm concentration, motility, and morphology into account (14). This study considers the direct relevance of motile sperm to the preparation of an insemination sample (selectively harvesting only motile sperm to place in the fertilization fluid around each oocyte). From the WHO definition, the lower limit of normal for semen samples is 60% (motile) of 20 $\times$ 10^6 spermatozoa/ml (density), i.e., 12 $\times$ 10^6 progressively motile forms/ml. Therefore from a range of 6 to 12 semen samples analyzed on each patient (4), husbands were categorized as follows:

Group 1: Severe oligospermia; $\leq 5 \times 10^6$ motile sperm/ml ($N = 10$).
Group 2: Moderate oligospermia; 6 to 11.5 $\times$ 10^6 motile sperm/ml ($N = 11$).
Group 3: Normospermic; $\geq 12 \times 10^6$ motile sperm/ml ($N = 27$).
Group 4: Abnormal morphology: >60% of spermatozoa exhibiting abnormal morphology ($N = 10$).

All cases in Groups 1, 2, and 3 were managed during a 4-month session of the program during which the overall pregnancy rate was 21% per laparoscopy. Cases excluded from consideration in this study were those undertaking IVF-ET for unexplained infertility or endometriosis, cases displaying poor follicular development following stimulation, cases with restricted access within the pelvis precluding the aspiration of follicles $\geq$1.6 cm, and cases using donor sperm. Five of the 21 oligospermic cases had an additional tubal factor, two in Group 1 and three in Group 2.

The 10 cases in Group 4 were managed over a 9-month period; four cases were concurrent with Groups 1, 2, and 3, and the additional six were the total subsequent cases arising in the ensuing 5 months when there were no significant differences in the overall fertilization and pregnancy rates.

The details of total sperm density with motility ranges and the proportion of abnormal forms within each group are documented in Table I. The proportion of motile spermatozoa considers only those with good to excellent progressive motility (4).

All semen samples were collected into a nontoxic polystyrene pyrogen-free sterile container (Medical Plastics, No. SJS 554250, Edwardstown, South Australia) in a room adjacent to the laboratory. PIVET Laboratory is incorporated in the operating theater suite of Cambridge Hospital and the semen collection room has an external entrance with a

Table I. Sperm Density, Motility Range, and Proportion of Normal Forms for Each Group Treated by in Vitro Fertilization

Group	Number	Sperm concentration × 10^6/ml [mean ± SE (range)]		% range (normal forms)
		Total	Motile	
1. Severely oligospermic	10	8.3 ± 2.34 (2.5–18)	3.2 ± 0.84 (1.5–5)	50–70
2. Moderately oligospermic	11	24.2 ± 1.92 (14–34)	9.2 ± 0.64 (6–11.5)	50–80
3. Normospermic	27	77.3 ± 7.25 (25–120)	48.4 ± 5.13 (14–90)	60–90
4. Abnormal morphology	10	34.8 ± 12.3 (10–80)	21.3 ± 9.2 (6–45)	10–40

double-door intervening hatchway (11). The room is kept warm (22–24°C) and sample containers along with specific instructions are available specifying directions for masturbation collection and avoidance of contaminating the container. Semen collections are placed immediately in the hatchway and a buzzer/light system alerts the staff so that samples can be received without delay and placed directly on a reciprocating rocker within the incubator at 37°C. A standard time of 30 min is allowed for liquefaction prior to analysis and the preparation of samples under constant conditions.

All patients had follicle growth stimulated with the details of monitoring, ovum aspiration, and embryo culture already described (11). The only difference in technique applied to oligospermic samples. Usually 0.4 to 1 ml of semen from normal males is used to collect a highly motile sample by the overlay technique (15) in order to achieve between 0.5 and 2.0 × 10^5 motile sperm in each insemination tube (Falcon No. 2003, Becton–Dickinson Co., Oxnard, CA) or multichamber culture dish (Nunc No. 134673, Kamstrup, Roskilde, Denmark) containing a single mature preovulatory oocyte. Depending upon the degree of oligospermia, between 1 ml and the entire sample is utilized. Semen is diluted 1 in 5 with modified Tyrode's solution (16) containing 7.5% deactivated maternal serum, and any viscous strands are dispersed by gentle pipetting before centrifugation at 200*g* for 15 min. The pellet is washed once more by centrifugation for 10 min, then overlayed with 4 ml of fresh fertilization medium. After 30 min of incubation at 37°C, a small aliquot is taken from the top of the overlay to count the number of motile sperm. If the concentration is greater than 1 × 10^6 motile sperm/ml, a 1-ml portion is carefully drawn from the top of the overlay. For the lower concentrations usually found in oligospermic samples, the entire 4 ml of the overlay is carefully removed without disturbing the pellet and centrifuged at 200*g* for 10 min. The supernatant fluid is removed and the concentrated spermatozoa are resuspended in variable volumes to achieve a density of about 1 × 10^6 sperm/ml. A 50 to 200-μl aliquot of this enriched motile sample is added to each fertilization tube to give a final concentration of between 0.5 and 2 × 10^5 motile sperm/ml (Fig. 1). The time of insemination is calculated to be approximately 40 to 42 hr following the hCG injection or the estimated onset of LH surge (4 to 8 hr from oocyte recovery).

Gametes are incubated overnight and examined 16 to 20 hr after insemination following dissection of the coronal coat with either fine needles or a finely drawn glass pipet. Fertilization is confirmed by the presence of two pronuclei in the cytoplasm of the oocytes and, where possible, a second polar body. Whether or not fertilization is demonstrated, all ova are washed once in the fertilization medium and transferred to a similar culture tube containing modified Tyrode's solution with 15% deactivated maternal serum. No attempt was made within the confines of this study to reinseminate unfertilized ova. Embryos and unfertilized ova are then cultured overnight and assessed the following morning for cleavage or fertilization. Embryo transfer is undertaken 44 to 48 hr after insemination, when embryos have usually developed to the four-cell stage. Pregnancies are diagnosed 16 to 19 days after laparoscopy by a rising concentration of β-hCG in the serum and confirmed about 5 weeks post-embryo transfer by the ultrasound detection of an intrauterine gestational sac.

All data were examined by chi-square analysis in 2 × 2 contingency tables applying Yates correction (17). Groups 2 and 3 were tested for homogeneity regarding fertilization rates and the proportion of

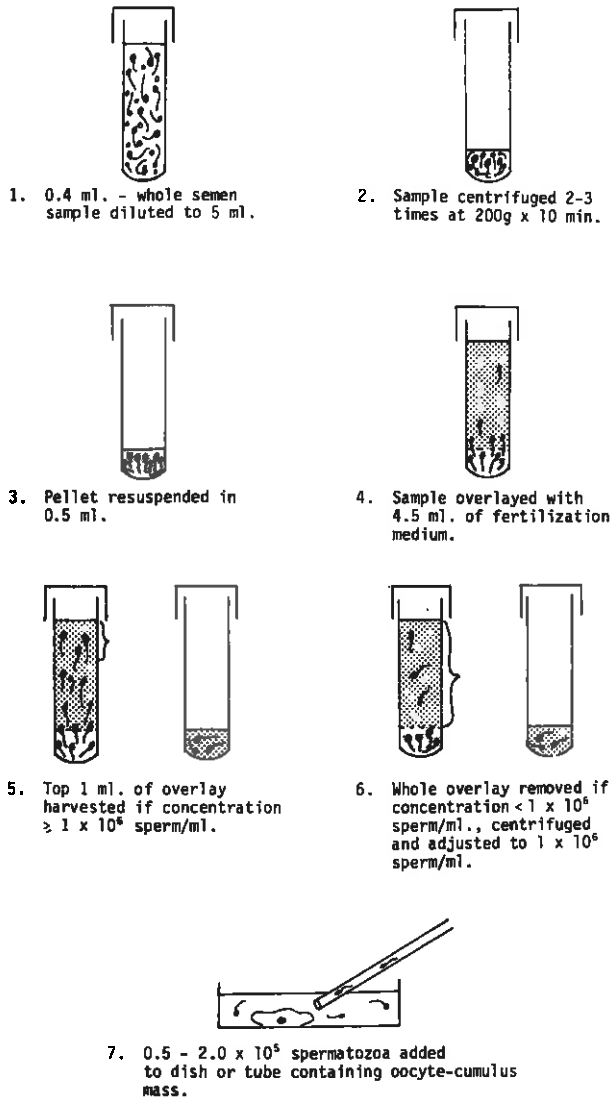

Fig. 1. The technique of sperm preparation for in vitro fertilization including the modifications applied to oligospermic samples.

four-cell embryos at the time of embryo transfer, then combined for the comparative analysis of data from Groups 1 and 4.

RESULTS

Overall 164 mature preovulatory oocytes (displaying a fully expanded cumulus mass at the time of aspiration) were inseminated and 122 were classified as fertilized by detecting either the pronuclear stage or subsequent cleavage (Table II). In all cases the concentration of motile sperm in the insemination tube was similar, in the range of 0.5 to 2.0 × 10^5 sperm/ml. However, the sperm of severely oligospermic males fertilized significantly fewer oocytes ($P < 0.001$) than sperm from the moderately oligospermic group, which demonstrated no significant difference from the normal samples of Group 3. Two of the oocytes from Group 1 which did not have pronuclei apparent at 16 to 20 hr postinsemination subsequently cleaved and were noted to be at the two-cell stage by 44 hr. In Groups 2 and 3 the pronuclear stage was recognized in all cases which subsequently cleaved. It is apparent that two oocytes in Group 1 exhibited delayed fertilization, however, the significance of the observation of reduced fertilization holds for both the pronuclear and the cleavage stages. Pregnancies were achieved in 2 of 10 cases exhibiting gross oligospermia, in 3 of the 11 cases with moderate oligospermia, and in 4 of the 27 normospermic cases. The rates were not significantly different. Four of the five oligospermic pregnancies and five of the seven normospermic group have now proceeded to term, delivering healthy infants.

Significantly fewer oocytes from those cases exhibiting abnormal sperm morphology (Group 4) had reached the pronuclear stage by 16 to 20 hr ($P < 0.001$), although the number of cleaving embryos noted at 44 to 48 hr was not significantly different from that of the normospermic group, implying delayed fertilization. This is further supported by the finding that significantly fewer embryos had reached the four-cell stage at the time of embryo transfer from Group 4 ($0.01 < P < 0.05$). One pregnancy was obtained in the abnormal oligospermic group but proved to be a blighted ovum, aborting at 6 weeks of gestation.

The categorization of spermatozoon anomalies into head, tail, or combined defects (Table III) indicated that most were in the combined group, and although the only case without head defects demonstrated normal pronuclear development with all embryos proceeding through to the four-cell stage at transfer, useful statistical comment cannot be provided.

The majority of couples (21) had demonstrable fallopian tube patency and were included in the IVF-ET program following at least four cycles of intrauterine insemination with husbands' washed sperm without conceiving. The persistent nature of the semen abnormality in these cases was therefore observed over several months. The presence of coexisting occlusive tubal disease (Table IV) did not influence the findings related to pronuclear stage development, total embryo development, or the

Table II. The Relationships of the Density of Motile Spermatozoa, Fertilization, and Embryo Development

Group	No. of motile spermatozoa × 10^6/ml	No. of patients	n/N[a]	Stage of embryo development at 44 hr			No. pregnant
				2 cells	3–4 cells	Total	
1	<5	10	14/34**	5/34	11/34	16/34	2
2	6–11.5	11	24/31	3/31	21/31	24/31	3
3	≥12	27	51/60	16/60	35/60	51/60	4
4[b]	≥6	10	19/39*	19/39	12/39**	31/39	1
Total		52	108/164	43/164	79/164	122/164	10

[a] n, number of ova with pronuclei at 16 hr postinsemination; N, number of ova inseminated.
[b] All cases demonstrated >60% abnormal morphology.
* $P < 0.001$.
** $0.001 < P < 0.01$.

proportion of embryos at the four-cell stage at embryo transfer.

DISCUSSION

Several extensive epidemiological studies have shown that sperm density is clearly linked to infertility when the concentration falls below 20 × 10^6 sperm/ml (1–3), although absolute infertility cannot be assured even with sperm densities of less than 5 million/ml. Problems associated with relating fertility to sperm density stem from the large intrasubject variation of volume, density, and total spermatozoon count and are related to ejaculation frequency. However, motility and normal morphology levels appear not to change significantly (5). We have related the rate of fertilization, embryo development, and pregnancy to the numbers of motile spermatozoa and separately categorized those patients displaying high levels of abnormal spermatozoa. The cases recruited in this study were those whose semen abnormality was regarded as the sole or a major factor causing infertility.

Table III. Effect of Elevated Abnormal Sperm Morphology on the Fertilization of Human Ova in Vitro

	No. of patients	Type of defect		n/N[a]	Stage of development at 44–48 hr		
		Head	Tail		2 cells	3–4 cells	Total
	1	−	+	3/4[b]	0/4	4/4	4/4
	7	+	+	13/28	17/28	5/28	22/28
	2	+	−	3/7	2/7	3/7	5/7
Total	10			19/39	19/39	12/39	31/39

[a] n, number of pronuclear embryos; N, number of ova inseminated.
[b] It is likely that one of the oocytes had proceeded beyond the pronuclear stage to syngamy (not clearly detectable by routine stereomicroscopy), as the four embryos had advanced beyond the two-cell stage at transfer.

The short incubation overlay technique described harvests only those spermatozoa with sufficiently strong progressive motility which can accumulate in the upper fraction of the tube. Spermatozoa which are nonmotile or weakly motile and those with tail defects which impede motility would not be expected to accumulate in the overlay fraction for subsequent insemination. The technique therefore tends to standardize samples and reduce potential variation in the composition of each sperm preparation. The initial reports on human IVF applied 1 × 10^6 motile sperm/ml in the insemination droplet (18) but subsequent studies have indicated that fertilization can be achieved with a motile sperm density as low as 1 × 10^4/ml (19). We have also noted this but find consistent high rates of fertilization only when the sperm density is in the range of 0.5 × 10^5 to 2 × 10^5 motile sperm/ml, which was the concentration range for all inseminations within this study.

The reduction in fertilization rates of the spermatozoa generated from chronic oligospermic males suggests that, when the mean initial concentration is low, greater variability exists in the fertilization potential between samples and that proportionately fewer motile spermatozoa are successfully able to interact with and penetrate human oocytes

Table IV. A Comparison of the Rates of Embryo Development in Groups with Variable Sperm Density and Morphology in Relationship to the Presence of Coexisting Occlusive Tubal Disease

	Nil	Tubal	Total
Group 1	10/26 (8)[a]	4/8 (2)	14/34 (10)
Group 2	18/24 (8)	6/7 (3)	24/31 (11)
Group 3	—	51/60 (27)	51/60 (27)
Group 4	11/16 (5)	20/23 (5)	31/39 (10)

[a] Number of patients in parentheses.

in vitro. While all spermatozoa may possess active forward motility at the time of preparation, perhaps fewer are able to complete capacitation and successfully penetrate the zona pellucida and oocyte. Whatever the underlying cause for the dysfunction in the male reproductive system, one manifestation of the pathology may be incomplete or abnormal sperm maturation. This could manifest itself in terms of an obvious morphological abnormality (20) or possibly in other less easily discerned functions such as membrane development and differentiation. It has been demonstrated in other studies with mice using suboptimal sperm concentrations that as the sperm density is reduced, fertilization rates are also reduced (21). Spermatozoa in this situation could be stimulated to complete the acrosome reaction by various physiological or pharmacological agents and, under these conditions, achieve ovum penetration (22,23). It would therefore be of interest to know if an increase in the fertilization rate may be obtained in human IVF for severely oligospermic males by subtle alterations in the culture conditions such as concentrations of albumin or pH variations.

The effect of gross morphological abnormalities on fertilization was examined in 10 cases. Within the group the fertilization rate was not reduced and the proportion proceeding to embryo transfer was the same as in the normospermic group. However, there was a clear delay in reaching the pronuclear stage and most embryos in this group were still at the two-cell stage (49%) at the time of embryo transfer, whereas the majority of embryos (65%) from the normospermic group was at the four-cell stage at the time of transfer (Table II). The series of 10 cases with abnormal sperm morphology has proven too small for detailed analysis of subgroups (Table III) but there is an apparent trend that the delay in fertilization may be more likely with those cases exhibiting proportionately higher head defects rather than tail defects. Such observations may relate to the overlay technique of sperm separation, which would be expected to collect only those spermatozoa able to move rapidly into the overlay during the short incubation period. Abnormal spermatozoa with solely tail defects would therefore be unlikely to appear in the insemination sample, which would contain morphologically normal spermatozoa only.

It is of course unknown if the fertilizing spermatozoon for each oocyte was morphologically normal or abnormal, but the data imply that most spermatozoa were defective in some aspect. Abnormal sperm morphology has been found to be a major reason for failure of samples in the zona-free hamster ovum penetration test (24). In comparative studies on the rate of ovum penetration in different strains of mice, differences were attributed to the rate at which spermatozoa complete capacitation (21,25). Delay in ovum penetration by spermatozoa from samples containing increased abnormal forms could possibly be explained by retarded reorganization in the plasma membrane overlying the acrosomal vesicle during capacitation (26). Alternatively, it has been reported in one study that patients with unexplained infertility demonstrated reduced acrosin activity of spermatozoa (27). Either way, this would manifest itself in impaired zona penetration and delayed fertilization.

One major advance in the assessment of the functional capacity of human spermatozoa has been the observation that capacitated, acrosome-reacted human spermatozoa can bind to and fertilize zona-free hamster oocytes, with subsequent development to the stage of nuclear decondensation (28). Several studies have attempted to validate the relationship between the zona-free hamster penetration test and infertility (6,29). Generally, it has been demonstrated that spermatozoa from infertile subjects and from oligospermic males exhibited evidence of reduced binding and penetration of the plasma membrane of the denuded hamster oocyte, with the major variables being the percentage of motile sperm (6) and sperm morphology (24). A correlation between zona-free hamster penetration tests and the fertilization of human oocytes in vitro has been documented (30,31) but the extrapolation of such results in relationship to human fertility requires caution for several reasons. First, the concentration of spermatozoa required for hamster ova penetration is of the order of 10 to 100 times that required for human ova penetration (32). A second factor is the ability of spermatozoa to negotiate the zona pellucida successfully. This is thought to be dependent upon capacitation, activated motility, and normal acrosin activity and can in part be assessed by the penetration of the zona pellucida of nonliving human oocytes (33,34). Although the fertilization rates reported here and by others (35) appear higher than may have been predicted from zona-free hamster assays, the results do support the observation that motile spermatozoa from oligospermic males exhibit a reduced fertilization potential (36) but only when the degree of oligospermia is severe.

The generation of pregnancies by IVF-ET in this study appears to relate directly to embryo development rather than the underlying cause of infertility (tubal or oligospermia) or the severity of oligospermia, although it is clear that in a larger series of grossly oligospermic patients, the proportion achieving fertilization will be reduced and hence the overall pregnancy rate in this group is expected to be less. However, this likelihood may be minimized by the generation of more oocytes using added human menopausal gonadotropin in the stimulation regime. Four of the five pregnancies derived from the oligospermic groups have delivered normal infants and the fifth is well advanced. The absence of abortions and the normal infants indicate that the process of sperm selection for IVF does not increase the potential for genetically defective embryos. The option of IVF-ET should be considered for cases of oligospermia and increases the potential for IVF to be applied as an effective therapy in nontubal as well as tubal causes of infertility.

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