

EFFICACY OF SPERM WASHING AND SWIM-UP METHODS IN AUGMENTATION OF TOTAL FUNCTIONAL SPERM FRACTION FOR DIRECT INTRAPERITONEAL INSEMINATION

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SUMMARY

Total Functional Sperm Fraction (TFSF) measurements were used to estimate the overall ability of males to achieve conception in 29 men from a group of infertile couples. TFSF is a more comprehensive criterion than the traditional semen measurements because it incorporates all important seminal parameters such as total cell count per ejaculate (TCE), percentages of normal morphology (N) and motility (M), and a measurement of progressive, straight-swimming spermatozoa (P) on a scale of 1-3. The application of sperm washing followed by swim-up procedure can improve TFSF while separating the motile, highly progressive spermatozoa from round cells, leucocytes, spermatids, contaminating microorganisms and anti-sperm antibodies. In a clinical trial of direct intraperitoneal insemination (DIPI), TFSF measurements were used for the evaluation of male fertility potential after sperm purification. TFSF measurements were utilized to standardize the number of active, highly progressive and morphologically normal spermatozoa for each individual case in every cycle of DIPI treatment. Results of the clinical trial showed that two out of six couples participating in DIPI were successful in achieving conception and having healthy babies.

Key Words: Sperm washing; swim-up; male infertility; direct intraperitoneal insemination.

INTRODUCTION

In the evaluation of the fertility potential of a male, the semen characteristics are of utmost importance. Conventional assessment of male fertility includes determination of sperm number, morphology and motility, usually by light microscopy and staining methods. Aitken and his coworkers (1) pointed out that these conventional criteria have poor predictive qualities by themselves and suggested the usage of time exposure photomicrography and the zona-free hamster egg penetration test. However, application of

these methods are not only expensive but are also time consuming. Furthermore, because of the variability of spermatozoa concentration, motility and vitality, semen analysis should be repeated two or three times (2,3). Recently published data indicate that the hamster egg test is not always a representative assay of a male's fertilizing potential (1,3-5). Unfortunately, although the same fluid is studied, terminologies, methodologies and standardizations vary from country to country if not from laboratory to laboratory. Thus, a new and improved technique was needed to eliminate the disparity among different laboratories.

This paper describes the use of a single new parameter called Total Functional Sperm Fraction (TFSF). The purpose of this investigation was to find an effective, reliable and objective means for measuring a male's fertility potential for our direct intraperitoneal insemination trials. Thus, we have used TFSF measurements to evaluate male's ability to achieve conception before and after sperm washing and swim-up applications.

MATERIALS AND METHODS

Total Functional Sperm Fraction Measurements (TFSFM):

Male fertility is measured by TFSFM, in order to ensure a representative sample of active spermatozoa ejaculated. TFSFM is calculated from the following formula:

$$TFSF = [TCE \times 10^6] \times N\% \times M\% \times P$$

TCE represents total cell count per ejaculate; N% represents percentage of normal morphology and M% represents percentage of motility per ejaculate at two hours at 37°C. Progressive motility of the spermatozoa is represented by P on a scale of 1-3; 1 is for sluggish, 2 is for motile but progressing slowly, 3

is for actively progressing straight swimming spermatozoa.

Trial Design:

Initially, 29 couples volunteered for the study between November 1987 to May 1988. The project was designed as a sequential trial comparing three groups of semen samples grouped according to their initial TFSF measurements. Initial TFSF values were compared with TFSF measurements after washing and swim-up application. Of 29 couples, 6 with longstanding infertility problems were selected both according to couple selection criterion and TFSF measurements obtained after washing and swim-up application.

Couple Selection:

For a couple to be admitted to this study, the man's semen had to satisfy at least two of the following criteria: more than 20×10^6 cells/ml, more than 30% motile sperm and more than 40% normal morphology. These limits include 72% of our reference population of men approaching the university clinic as potential patients for DIPI treatment. Furthermore, the prospective couple must have been trying to conceive at least two years. Women must have been ovulating or alternatively, were capable of stimulation by estrogens. Ovulation was determined either by serum progesterone concentrations of greater than 20nmol on the 17th day after luteinizing hormone (LH) surge or by positive follicular development. The latter is monitored by ultrasound from day 9 onwards till the day the follicle was found to have a diameter of 18-19mm. In addition, women had to have patent normal fallopian tubes as judged by a laparoscopic tubal dye insufflation test.

Material Collection:

Semen intake:

Male patients were advised to abstain from ejaculation for 3 days before the laboratory visit. Semen samples were obtained by masturbation in a sterile petri dish under clean conditions.

Liquefaction and Measurements:

Semen samples were allowed to liquefy for 30 min at room temperature; 100ml aliquots were taken for direct counting by light microscopy and for bacteriological examination. Before starting the purification initial semen indices, i.e. viscosity, sperm density, percentages of motility (M) and normal morphology (N), progressive motility (P) on the scale of 1-3, total volume of the ejaculate (TCE), fructose content, pH and liquefaction time were measured. Subsequently, remaining samples were used in the washing and swim-up procedures. Purified samples' TFSF measurements (i.e. sperm density, M%, N%, TCE, P, and pH) were evaluated before the DIPI treatment for each application.

Media preparation:

Washing media: Minimum essential media (MEM) with Earl's salt containing L-Glutamine and 26mM sodium bicarbonate (GIBCO, Grand Island, NY) was supplemented with 8% male patient serum. The serum was heat inactivated and sterilized by filtration as previously described (6). For each application a fresh serum preparation was used. Benzyl Penicillin-G and Streptomycin sulfate (100 IU/ml), 0.33mM pyruvic acid, sodium salt (Sigma, St.Louis, MO) were also added to media, which was sterilized by filtration and was stored at 4°C for 48 hours. The osmolality of the media was adjusted to 280mOsm/l and the pH was kept constant at 7.2. When Ham's F-10 media was used, it was buffered with 25mM HEPES (Sigma, St.Louis, MO). Each time fresh preparation was used except when DIPI was applied to the same patient on two consecutive days. In patients exhibiting sperm antibodies in their semen usage of the patient serum was omitted. Instead human bovine albumin (Sigma, St.Louis, MO), 16µg/ml, was used.

Swim-up Media: The same media (MEM/Ham's F-10) with all above additions were used for swim-up procedures with a serum concentration of 10%. HEPES concentration was 10mM, the pH was adjusted to 7.4 and calcium lactate, 20mM, was added. The media was sterilized by filtration. Osmolality was kept at 280mOsm/l.

Sperm Washing:

After the initial measurements, total liquefied semen was used for the first wash. Over one volume of semen, three volumes of washing media was added drop by drop in gammasterilized Becton and Dickinson (Mountain View, CA) disposable tubes. The fluid was centrifuged at 200 g for 5 min at 20°C. Supernatant was decanted (after checking for the presence of motile sperm) and the pellet was resuspended in 9ml of media for the second wash and was centrifuged for a further 5 min at 200g at 20°C. Again supernatant was checked for the presence of motile sperm and was decanted. The pellet was used for further purification by swim-up procedures.

Swim-up procedure:

Pellets from the second wash were underlaid gently by 0.5ml of swim-up media. Without disturbing the pellet, the tubes were transferred to a 37°C, 5% CO₂ and 95% air mixture incubator for 30 min. After the incubation, the cloudy top layer containing highly motile, morphologically normal sperms were removed and checked for TFSF measurements. If the yield was low, the layering and incubation steps were repeated until a clear top layer was obtained. Total TFSF measurements were calculated by pooling top layers together.

RESULTS

Augmentation of TFSF measurements:

Among the infertile couples, 29 male patients were randomly selected and their TFSF measurements were analyzed to evaluate the benefits of washing and swim-up procedures. Patients were grouped according to their initial TFSF values.

The first group contained 8 patients with less than 20×10^6 cells/ml. The group's initial mean TFSF was 8.5×10^6 (Table I). Application of the washing and swim-up methods resulted in the decrease of initial sperm density from 14×10^6 to 8.5×10^6 cells/ml with a sperm recovery of 61%. However, TFSF was in-

morphology, 90%, and progressively swimming sperm, $P=3$ (Table I). When analyzed statistically, the difference between means of TFSF before and after swim-up was -8.25×10^6 , SEM 1.86, $p<0.01$ which are highly significant values for functional sperm counts. Analysis of motility % differences by the same method showed a difference of -24.5% M, SEM 4.31 and $p<0.01$, indicating again highly significant increases in motility (Table I).

The third group, contained 9 cases with larger than 80×10^6 cells/ml sperm density. Initial TFSF of the group was 204×10^6 (Table I). After washing and

Table I. Evaluation of TFSFM on 29 oligospermic cases before and after the application of sperm washing and swim-up methods (measurements represent mean and standard deviation of 3 experiments).

Patient groups/Treatment	Density* cells/ml	M% **	N%	P(1-3)***	Vol. ejaculate	TFSF****	Sperm recovery %
Group 1 (< 20×10^6 cells/ml) n=8							
Pretreatment	14×10^6	28	70	1	3.10ml	$8.5 \pm 5.5 \times 10^6$	N/A
Posttreatment	8.5×10^6	48	85	2	N/A	$32.2 \pm 4.7 \times 10^6$	61
Group 2 ($20-80 \times 10^6$ cells/ml) n=12							
Pretreatment	51×10^6	43	76	1	3.95ml	$66 \pm 16.4 \times 10^6$	N/A
Posttreatment	42×10^6	65	90	3	N/A	$291 \pm 16.5 \times 10^6$	83
Group 3 (> 80×10^6 cells/ml) n=9							
Pretreatment	120×10^6	57	89	1	3.35ml	$204 \pm 30 \times 10^6$	N/A
Posttreatment	95.8×10^6	81	96	3	N/A	$250 \pm 16.9 \times 10^6$	80

*Samples are collected after 72 h abstinence.

**Samples were evaluated at 2 h, at 37°C

***Reference 17.

****TFSF = TCE[10^6] \times M% \times N% \times P

creased from 8.5 to 32.2×10^6 after purification (Table I). When both TFSF values were analyzed on the basis of difference between means paired observation t-test, the mean TFSF was 4.75×10^6 , the standard error of the mean (SEM) was 1.12 and $p<0.01$. This indicates not only highly significant improvements of TFSF, but also its contributing parameters (i.e. 48% M, 85% N, $P=2$) were highly augmented (Table I). Analysis of M% difference before and after application of washing and swim-up by the same statistical method showed that the difference between means of M% was -18%, SEM 6.37 and $p<0.01$, indicating significant increases in the motility.

The second group contained 12 patients with sperm densities of $21-80 \times 10^6$ cells/ml, had a mean TFSF value of 66×10^6 (Table I). After washing and swim-up application sperm recovery was 83%, leading to a lower sperm density (42×10^6 cells/ml) but a higher TFSF value of 291×10^6 . This indicated highly significant improvements in motility, 65%, normal

swim-up application. TFSF was increased to 250×10^6 , indicating 95.8×10^6 cells/ml sperm density, 81% M, 96% N and $P=3$ with 80% sperm recovery. When analyzed statistically, the difference between the means of TFSF was found to be -24.94×10^6 with SEM 5.51 and $p<0.01$. Analysis of the motility by the same test showed a mean of -23.78%, SEM 3.13 and $p<0.01$. Negative notation represents an increase in the statistical analysis.

The data presented indicate that in all cases there is significant improvement in both motility and TFSF after purification. Low sperm recoveries resulted from starting material that contained more round cells, leucocytes, cell debris and spermatids than normal spermatozoa. Highly leucocyte-contaminated semen from one patient (L.K.) is shown in Fig. 1 (A and B); the presence of cell debris can be observed. After two consecutive washes followed by swim-up application, a clear difference was obtained as seen in Fig.

2. Data presented so far indicate that methods applied were successful in eliminating contaminating elements from active, fully functional spermatozoa. Previously, we have reported the usage of a similar purification methods in IVF-ET and GIFT treatments (6,7). However in the DIPI trial, methods were improved. The swim-up media was supplemented with 20mM calcium lactate and 0.33mM sodium pyruvate, complimented with/without 10% male patient's serum according to the particular case.

Clinical Application of TFSF Measurements:

After the analysis of TFSF values, 6 couples with long standing infertility problems [duration of infertility was between 2-4, mean 8±4 years] were chosen for clinical trials of TFSF in DIPI treatments. The treatment should have been continued for at least 6 cycles before evaluation and most of the patients were not readily committed. However, the 6 couples chosen for the trial were representative of the three groups

demonstrated head to tail agglutination, was also successfully treated on the fifth cycle and had a healthy child (8). Two couples completed 3 cycles, the last 2 couples had only 2 cycles of the treatment with no pregnancy, and none wanted to complete the trial.

DISCUSSION

Outcome of the clinical trial suggests that TFSF measurements represent a realistic evaluation of the fertility potential of a male when selecting a couple for their cervical factor, oligospermia and unexplained infertility treatments. Endocrinological, gynecological and obstetrical aspects of the DIPI trials were published elsewhere (8).

Furthermore, the ejaculate of every man contains a variable amount of round cells, provided the period of sexual abstinence does not exceed 5 days, the semen sample should not contain more than one million peroxidase (+) leucocytes and/or peroxidase (-) spermatogenic cells or prostatic cells for successful

Table II. Before and after application of sperm washing and swim-up, evaluation of conventional parameters and TFSFM of male participants in DIPI trial (measurements represent mean application in each case).

Patients/n*	Vol. Ejaculate	pH	TCE	Density Cells/ml	N%	M%	P(1-3)	Agglutination	% Contamination	TFSFM	Pregnancy
Y.M. pretreatment (12)	1.8ml	8.6	140 × 10 ⁶	78 × 10 ⁶	89	69	1	head/tail	0.1	86 × 10 ⁶	—
posttreatment	—	7.4	—	62 × 10 ⁶	100	98	3	none	none	182 × 10 ⁶	+
K.P. pretreatment (6)	2.7ml	8.0	132 × 10 ⁶	50 × 10 ⁶	56	30	1	head/tail	10	23 × 10 ⁶	—
posttreatment	—	7.4	—	18 × 10 ⁶	78	48	2	none	0.1	36 × 10 ⁶	—
Z.S. pretreatment (6)	3.8ml	8.0	108 × 10 ⁶	28 × 10 ⁶	76	37	1	head/tail	4	31 × 10 ⁶	—
posttreatment	—	7.4	—	18 × 10 ⁶	90	68	3	none	none	126 × 10 ⁶	+
L.K. pretreatment (8)	2.0ml	7.5	230 × 10 ⁶	115 × 10 ⁶	80	43	1	head/head	8	79 × 10 ⁶	—
posttreatment	—	7.4	—	79 × 10 ⁶	98	87	3	none	0.1	404 × 10 ⁶	—
A.K. pretreatment (5)	4.0ml	9.0	420 × 10 ⁶	105 × 10 ⁶	80	54	1	head/head	0.1	181 × 10 ⁶	—
posttreatment	—	7.4	—	80 × 10 ⁶	90	81	3	none	none	787 × 10 ⁶	—
U.B. pretreatment (4)	4.0ml	8.5	248 × 10 ⁶	57 × 10 ⁶	86	36	1	head/head	0.1	80 × 10 ⁶	—
posttreatment	—	7.4	—	31 × 10 ⁶	1	6	2	none	none	173 × 10 ⁶	—

described above and had mostly male subfertility and/or cervical factor and unexplained infertility problems. All carried the necessary minimal requirements of our couple selection criterion. The mean age of the patients was male 38±4 years and female 34±5 years. Female patient case history showed at least one or two abortions in the early years of marriage, with the exception of U.B. (8). Initial TFSF values and benefits of the washing and swim-up application were demonstrated in Table II. One couple, Z.S., who belonged to the low sperm density group and demonstrated head to head sperm agglutination, was successfully treated on the third cycle and had a healthy child. Another couple, Y.M., who belonged to the moderately normal sperm density group and

conception (i.b.3). The application of our method clearly demonstrates that these cells, usually indicating abnormalities such as inflammatory reaction of the adnex glands or in the case of spermatids and spermatogonia, tubular dysfunction with sloughing of immature cells, can easily be separated from functional sperm by twice washing followed by swim-up application. Significant bacteriospermia is not correlated to tapering heads, coiled tails, or decreased motility; these alterations can occur in both the absence or presence of infection as well as in other pathological conditions such as varicocele and idiopathic oligozoospermia (i.b. 3). Purification of the semen by sperm washing and swim-up procedures eliminated not only bacterial contaminants but also

sperm with morphological and motility abnormalities. It is very important that we were capable of cleaning the microorganisms and abnormal forms of spermatozoa from the preparation because in the application of DIPI, they are the primary cause of intrauterine and pelvic infections and failure of insemination.

There have been many reports on the application of various sperm washing and swim-up methods and their application in infertility treatments (9-13). Most of them pointed out the benefits of the methods for the purification from bacteriological contamination and elimination of anti-sperm antibodies. Presented data show that we were able to overcome head to head and head to tail agglutination of sperm impairs motility. Spermatozoa cleared of seminal fluid was also cleared of antibodies by resuspension in swim-up media without patient serum, which might have contained circulating antibodies. These results imply that in vitro constructed media components are very important in the success of infertility treatment.

Variations exist between the ejaculates of individuals as well as between the different ejaculates of the same individual in terms of traditional semen parameters. The usage of TFSF can easily eliminate these discrepancies. The number of the spermatozoa, normal morphology, semen pH and osmolarity can be adjusted for optimized effect during washing and swim-up procedures performed in the appropriate media. Furthermore, motility rates can be improved by the addition of pyruvate 0.33mM and lactate 20mM into the swim-up media. The selective media used in these experiments also provided the optimal pH, 7.4, and osmolarity, 280 mmol, which are important for the progression of normal spermatozoa. Moreover morphology and motility are highly consistent parameters (14-16) that have significant correlations with fertilizing capacity in unexplained infertility patients (17) and of all the conventional parameters of semen quality, they appear to be the most sensitive indicators of sperm function. Thus, the TFSF measurements used in this study indicate a more comprehensive criteria than traditional semen parameters because they emphasize the value of morphologically normal, motile, straight-progressing spermatozoa better than each of the individual parameters used in other studies. Furthermore, we can optimize the sperm number for each application of DIPI. Thus, the usage of TFSF measurements for the evaluation of a male's fertility potential gives us the opportunity to characterize and optimize the parameters for each trial.

Infertility treatments usually last for a long period of time. In this period, there are several attempts at conception and any method which yields comparable, reliable results leading to increased fertility could be very valuable. The data presented here indicate that spermatozoa cleaned from debris and contamination and improved as measured by TFSF by washing and swim-up techniques may be useful for DIPI, IVF-ET and GIFT treatments of infertility.

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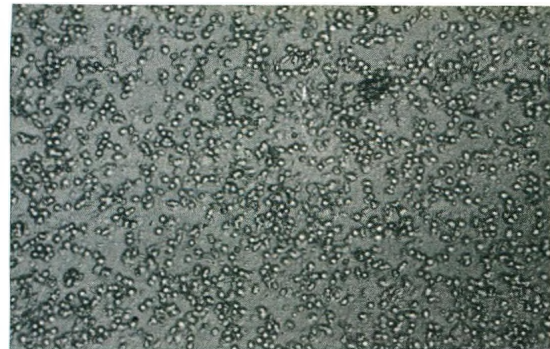


Figure 1a.

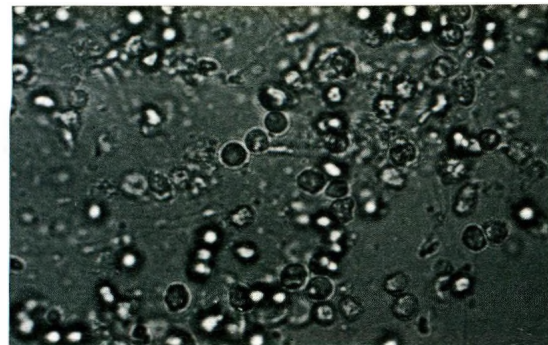


Figure 1b

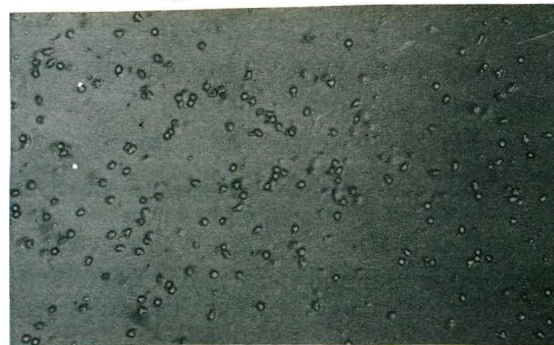


Figure 2.

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