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An Analysis of pre and post-Processing Semen Parameters at The Time of Intrauterine Insemination; and The Confounding Effects of Total Motile Sperm Counts on Pregnancy Outcome: A Prospective Cohort Study

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Abstract

Background: This study aims to determine whether pre or post-processing semen parameters obtained during intrauterine insemination (IUI) predict pregnancy when controlling for confounding effects.

Materials and Methods: A prospective cohort study of 2231 semen analyses was conducted at McGill University of IVF center. Any couples who underwent IUI with partner sperm, over a 2.5-year period, were included. Controlled ovarian stimulation was done with Clomiphene Citrate, Letrozole, or Gonadotropins. Statistical analysis was performed using t tests, two types of stepwise logistic regression, and stepwise discriminant analysis. A comparison of pre and post-processing semen parameters was undertaken to determine the probability of pregnancy.

Results: There were significant differences between pregnant and non-pregnant women in post-processing concentration (P=0.043), post-processing total motile sperm count (TMSC) (P=0.049), and post-linearity (P=0.012). However, when variable out-of-the-equation logistic regression or discriminant analysis, which controls for confounding effects between variables, were used, the findings were no longer significant. It was statistically proven that when a variable in the equation logistic regression was employed, post-processing concentration (P=0.005) and post-processing TMSC (P=0.009) remained reliable predictors of pregnancy.

Conclusion: Two of three prediction models suggested that TMSC's relationship with pregnancy is due to confounding factors. One model maintained the validity of the TMSC. While TMSC has always been studied as an important predictor of insemination pregnancies, this finding may be due to confounding effects between semen parameters and therefore requires further investigation as to this relationship.

Keywords: Artificial Insemination, Pregnancy, Semen Analysis

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Introduction

The Centers for Disease Control and Prevention (CDC) defines infertility as the inability to conceive after a year of unprotected intercourse (1). Couples who present for an infertility evaluation complete a semen analysis as part of their initial evaluation (2, 3). Of all factors, the most important is felt to be the total motile sperm count (TMSC) when it comes to predicting pregnancy (3, 4). However, the value of TMSC has become controversial (5) with other studies not finding a relationship with intrauterine insemination (IUI) outcomes. Since semen processing can alter the specimen, the post-processing parameters are hypothesized to be more prognostic than the pre-processing values in predicting the likelihood of pregnancy (4, 6). Most studies have found TMSC to predict pregnancy outcomes (7, 8). Furthermore, a systematic review published in 2014 by Ombelet et al. (8) analyzed the literature published and established that the TMSC was a tool with substantial discriminatory ability. In a longitudinal cohort study conducted in three Dutch hospital sites, it was found that TMSC was a better correlator of spontaneous pregnancy than the 2010 World Health Organization's (WHO) classifications. Furthermore, the article's data

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suggests that TMSC should be used as an indicator when defining the severity of male infertility as it is a more exact parameter (3). Similarly, a 2016 study found that TMSC was more predictive than the WHO 2010 cut-off values for pregnancy outcomes in couples undergoing inta-cytoplasmic sperm injection (ICSI) (9). A 2021 study found that in mild male factor infertility, TMSC is related to pregnancy outcomes (10).

However, a retrospective analysis from China found that a decrease in TMSC did not affect pregnancy outcomes at IUI (11). Other recent studies have also failed to confirm this relationship (5). Why some studies find a value in the TMSC and others do not about pregnancy parameters is unknown. Few if any studies have evaluated the confounding effects of other semen analysis parameters on the TMSC, which may be an explanation for the conflicting results in the literature as related to TMSC and pregnancy outcomes. This study was conducted to further analysis the effect of all analyzed parameters on pregnancy results, and whether pre or post-processing semen analysis results are in fact more predictive of pregnancy.

Materials and Methods

Study design

In this a prospective cohort study, all the pre and post-processing semen analysis results were performed before the insemination at the institution over 2.5 years and were prospectively enrolled in this database to be studied. This amounted to 2231 semen analyses at the time of IUI from 2227 patients. Fresh partners' semen was included in the analysis. Donor IUI semen results were excluded. No patients during this period opted out of the analysis.

Participants

Infertility was defined as a minimum of 1 year of unprotected intercourse without achieving pregnancy per the Centers for Disease Control (1). The duration of infertility amongst the couples ranged from one to seven years. All female partners required unilateral fallopian tube patency, which was tested by either hysterosalpingography or laparoscopy with chromopertubation. No subjects had untreated intra-cavitary lesions including polyps or fibroids, hydrosalpinges in-situ, thyroid, or prolactin abnormalities. The couples' indications for IUI included: male factor subfertility, ejaculation dysfunction, endometriosis, ovulatory dysfunction, and unexplained infertility. The women in the study range between 21- 42 years old. The fertility workup among the couples included the following for women: complete medical history, physical exam, complete blood count, thyroid function test, serum hormone levels on day 2-5 of their spontaneous or progesterone-provoked menstrual cycle (estradiol, total testosterone, prolactin and Follicle Stimulating Hormone), a transvaginal ultrasound, and hysterosalpingography on cycle day 6 to 11 or a laparoscopic demonstrating tubal patency. The male fertility workup included: a complete medical history, a physical exam, serologies, and a semen analysis.

Subjects with less than 5 million total motile sperm count or teratozoospermia (<4 % normal strict morphology), long histories of infertility greater than 3 years duration, stage 3 or 4 endometriosis, or histories suggestive of extensive pelvic adhesions were recommended to go to *in vitro* fertilization (IVF) and avoid insemination however, this was not mandatory. Table 1 outlines the participants' inclusion and exclusion criteria.

Table 1: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
One year of infertility	Untreated intra-cavitary lesions
Unilateral/bilateral fallopian tube patency	Uterine polyps or fibroids
IUI indications: male factor subfertility, ejaculation dysfunction, endometriosis, ovulatory dysfunction, and unexplained infertility	Thyroid or prolactin abnormalities
Women between 21-42 years old	

Procedure

The semen analysis was performed in conjunction with the WHO laboratory manual for the examination and processing of human semen - 5th ed (12).

The IUI procedure required a fresh semen collection, in which individuals were asked to refrain from ejaculation for two days before collection of the specimen, but not more than four days. Specimens were produced by masturbation in a collection room next to our laboratory or at the patient's homes. If semen collection was performed in the patients' home it needed to be delivered to the clinic no more than 30 minutes later, to maintain fresh and viable semen.

The semen analysis was performed in the following manner. Ejaculated sperm were permitted to liquefy before initial analysis. Liquefied semen was mixed before being placed on a standard count slide (Leja Products BV, Nieuw-Vennep, the Netherlands). The loaded slide was placed on a 37°C stage of an integrated visual optical system (IVOS) computer-assisted semen analyzer (Hamilton Thorn Biosciences, Beverly, MA) for every analysis. A minimum of three random fields were checked for each analysis. After density gradient separation of Pure Sperm (Nidacon, Molndol, Sweden), specimens were washed and concentrated to approximately 0.5 ml, and an aliquot of the concentrate was analyzed by computer-aided sperm analysis (CASA) ("post"-assessment). The results of the CASA were validated by manually examining a slide of sperm under high-power field microscopy by an andrologist. Intra and inter-assay coefficients of variation were below 10%.

For any questions related to the measurement or meaning of the parameters by the Hamilton Thorn CASA system, we refer you to https://www.hamiltonthorne.com/index.php/71-documentation/manuals.

Semen processing was performed in the following manner. Following liquefaction and semen analysis ("pre"), a maximum of 4 ml of raw semen is placed on a differential density gradient column consisting of 1 ml, 40%, and 1 ml, 80% Pure Sperm (Nidacon, Molndol, Sweden). The column was centrifuged for 20 minutes at 350 × g. Following centrifugation, the 40% layer and the seminal plasma fractions were removed from the test tube, and the 80% layer was left. About 6-8 ml of sperm washing medium and 5% human serum albumin (HAS, Cooper Surgical, USA) were mixed with the 80% layer and centrifuged for 10 more minutes at 550 × g. After centrifugation, the sperm pellet was recalibrated to contain about 0.5 ml, and a portion was analyzed for the "post" assessment.

Strict morphology was not included because to perform this analysis part or all the specimen needs to be killed and stained and since this specimen was being used to perform the insemination, strict morphology was not analyzed.

Specimens with levels of leukocytes indicative of an acute infection were not inseminated. Couples in this case were informed to undergo testing for the cause of this infection.

A positive pregnancy test (A pregnancy), and not a clinical pregnancy or a live birth, was selected as the outcome measured as "success". A positive pregnancy test is described as an increased level of β-human chorionic gonadotropin (β-hCG), which is released during the early weeks of pregnancy. In contrast with a clinical pregnancy, which is confirming the pregnancy visually- by ultrasound. A positive pregnancy test was felt to reflect the sperm's capacity to fertilize the oocyte. The presence of an ultrasound confirmed clinical pregnancy would have been modulated by aneuploidy and other genetic abnormalities, as well as endometrial factors, which are sperm independent. Had we selected live birth as the outcome measured, it would have been further modulated by the maternal environment and pregnancy complications. As such neither live birth nor clinical pregnancy was selected as the outcome of interest.

The goal was to understand the role of semen parameters on pregnancy outcomes, irrespective of other female or male factors, and help the physician guide the patients in terms of pregnancy outcomes at the time of insemination. Although factors such as male age, female age, and ovarian reserve parameters may play a role in pregnancy outcomes at insemination, we did not attempt to analyze the impact of these variables. When the couple undergoing IUI, asks the physician what the likelihood of pregnancy is, the physician does not consider any factors at that time beyond the quality of the sperm. As such, this study will help physicians counsel patients, and educate them on pregnancy outcomes based on our findings.

The IUI procedure was conducted 24 hours after a urinary lutenizing hormone (LH) surge, or 36 hours after β-hCG injection (10,000 IU, Merck and Co, USA, or Ferring Pharmaceuticals, USA or Ovidrel 250 mcg, Merck-Serono, USA). β-hCG was administered when the transvaginal ultrasound measured the largest follicle diameter to be ≥ 18 mm. Next, insemination was conducted in a sterile manner. A flexible plastic catheter was inserted into the female, while she lay in the dorsal lithotomy position. Post-insemination, the patient lay down for about ten minutes, to allow gravity to help the sperm move upwards through the uterus. Serum β-hCG levels were drawn from the patient around 16 days post-IUI, in order to establish pregnancy status and a baseline β-hCG level. A positive pregnancy was defined as β-hCG higher than 10 mIU/ml.

Statistical analysis

All statistical analyses were performed using the statistical package for Social Sciences 23.0 (SPSS Inc., Chicago, IL). Continuous variables were assessed for normal distribution using the Kolmogorov-Smirnov test. Any non-parametric distributions were logarithmically transformed to obtain a normal distribution for analysis (Table 2). Results are reported as mean value ± standard deviation (SD). Discriminators (statistical determinates as measured by relevant variables) of pregnant versus not pregnant among the pre and post-processing semen analysis parameters were assessed using Student's t test, the two types of logistic regression analysis and stepwise discriminant analysis. Both stepwise logistic regression and stepwise discriminant analysis were used since these are different techniques that could verify the results of the other analysis. Two types of stepwise logistic regression were employed, the variable in the equation method and the variable out of the equation method. The confounding effects controlled for were all parameters listed in Table 2. Approval from Stanford University's committee for the protection of human research subjects was obtained for the collection and analysis of this study's data. It should be noted that the variable out-of-the-equation method of logistic regression does not generate an odds ratio or a confidence interval, and only a P value is provided.

A power analysis was performed to determine whether adequate study size was present. The values to calculate were the means and standard deviation obtained for the post-processing TMSC. MU1 33, MU2 29, sigma was 2, with a 5% alpha and 80 percent beta, and the number of IUI needed for significance was 40. Therefore 2231 IUI was an adequate enrollment. There is no technique for power analysis for stepwise logistic regression or discriminant analysis.

Table 2: Comparison of pre and post-processing semen analysis parameters in pregnant and none pregnant patients by Student's test

Parameters	Pregnant	Not pregnant	P value
Initial volume (ml)	2.9 ± 1.5	3.0 ± 1.6	0.492
Initial concentration (M/ml)[a]	56 ± 43	52 ± 42	0.086
Initial percent motile (%)	49 ± 21	48 ± 22	0.319
Initial concentration motile (M/ml)[a]	32 ± 35 (not transformed values)	31 ±42 (not transformed values)	0.070 (from Log transformation)
Initial total motile sperm count (M)[a]	89 ± 107	84 ± 101	0.349
Initial progression (U/seconds)	44 ± 9	45 ± 10	0.171
Initial path speed (U/seconds)	76 ± 19	78 ± 35	0.272
Initial linearity (0-100)	58 ± 9	58 ± 9	0.460
Initial lateral head displacement (U)	3.4 ± 2.8	3.4 ± 2.5	0.920
Initial velocity average path (U/seconds)	52 ± 11	53 ± 12	0.240
Post volume (ml)	0.51 ± 0.08	0.52 ± 0.16	0.684
Post concentration (M/ml)[a]	75 ± 81	67 ± 73	0.043[a]
Post percent motile (%)	72 ± 25	72 ± 24	0.721
Post concentration motile (M/ml)[a]	64 ± 78	58 ± 70	0.089
Post total motile sperm count (M)[a]	33 ± 45	29 ± 37	0.049[a]
Post progression (U/seconds)	63 ± 15	63 ± 16	0.453
Post path speed (U/seconds)	113 ± 29	113 ± 30	0.908
Post linearity (0-100)	58 ± 28	57 ± 8	0.012[a]
Post lateral head displacement (U)	5.0 ± 5.7	4.8 ± 3.3	0.349
Post velocity average path (U/seconds)	73 ± 19	73 ± 19	0.971

Data are presented as mean ± SD. M; Million and [a]; <0.05 statistically significant when using t tests which do not control for confounding effects of the other variables analyzed, post-processing concentration (P=0.043), post-processing total motile sperm count (P=0.049), and post linearity (P=0.012) all are significant discriminators between the pregnant and not pregnant group. While preprocessing total motile sperm count and sperm concentration, among the other factors failed to be related to pregnancy outcome.

Ethical considerations

The Stanford University Committee for the Protection of human research subjects' approval has been obtained for the collection and analysis of this data (IRB 284365). Patients' written consent was obtained.

Results

All continuous variables were normally distributed except for the initial concentration of motile sperm which was logarithmically transformed for all statistical analysis.

Twenty-two percent of IUI's achieved a pregnancy. A comparison using the student's t test of the semen parameters in the group that conceived and the group that did not can be seen in Table 2. It can be noted, that when using the t test which does not control for confounding effects of the other variables analyzed, post-processing concentration, post-processing total motile sperm count, and post linearity all are significant discriminators between the pregnant and not pregnant group.

Stepwise discriminant analysis was performed since it is a technique to detect differences that predict inclusion in one of two groups while controlling for confounding effects (Table 3). The minimum F to enter the computation was 3.84 which is the minimum value to result in a statistically significant result. None of the variables reached the minimum F which could result in a statistically significant comparison.

Table 3: Evaluation of the ability of pre and post-processing semen analysis parameters to predict pregnancy by stepwise discriminant analysis

Parameter	F to enter
Initial volume (ml)	0.16
Initial concentration (M/ml)[a]	2.75
Initial percent motile (%)	0.74
Initial concentration motile (M/ml)[a]	0.31
Initial total motile sperm count (M)[a]	1.22
Initial progression (U/seconds)	1.91
Initial path speed (U/seconds)	1.22
Initial linearity (0-100)	0.67
Initial lateral head displacement (U)	0.45
Initial velocity average path (U/seconds)	1.39
Post volume (ml)	0.17
Post concentration (M/ml)[a]	3.11
Post percent motile (%)	0.001
Post concentration motile (M/ml)[a]	2.34
Post total motile sperm count (M)[a]	3.35
Post progression (U/seconds)	0.56
Post path speed (U/seconds)	0.50
Post linearity (0-100)	1.86
Post lateral head displacement (U)	0.57
Post velocity average path (U/seconds)	0.001

For the Stepwise discriminant analysis, the minimum F to enter the computation was 3.84 which is the minimum value to result in a statistically significant result. As can be noted above, none of the variables reached the minimum F which could result in a statistically significant comparison. In other words, none of the sperm parameters predicted a pregnancy using discriminant analysis. M; Million and [a]; <0.05 statistically significant.

Table 4: Evaluation of the ability of pre and post-processing semen analysis parameters to predict pregnancy by stepwise logistic regression analysis

Variable out of the equation						Variable in the equation							
Variable	P value	Variable	P value	Variable	P value	OR	95% CI		Variable	P value	OR	95% CI	
							Lower	Upper				Lower	Upper
Initial volume (ml)	0.692	Post volume (ml)	0.680	Initial volume (ml)	0.991	1.001	0.906	1.086	Post volume (ml)	0.063	0.055	0.003	1.132
Initial concentration (M/ml)[a]	0.097	Post concentration (M/ml)[a]	0.080	Initial concentration (M/ml)[a]	0.008	1.014	1.004	1.024	Post concentration (M/ml)[a]	0.065	1.01	0.999	1.021
Initial percent motile (%)	0.388	Post percent motile (%)	0.972	Initial percent motile (%)	0.430	1.009	1.000	1.018	Post percent motile (%)	0.839	1.001	0.994	1.008
Initial concentration motile (M/ml)[a]	0.154	Post concentration motile (M/ml)[a]	0.129	Initial concentration motile (M/ml)a	0.008	0.981	0.967	0.995	Post concentration motile (M/ml)[a]	0.005	0.975	0.958	0.992
Initial total motile sperm count (M)[a]	0.267	Post total motile sperm count (M)[a]	0.069	Initial total motile sperm count (M)[a]	0.653	1.001	0.998	1.003	Post total motile sperm count (M)[a]	0.009	1.031	1.008	1.055
Initial progression (U/seconds)	0.180	Post progression (U/seconds)	0.460	Initial progression (U/seconds)	0.541	0.988	0.950	1.027	Post progression (u/sec)	0.190	1.016	0.992	1.039
Initial path Speed (U/seconds)	0.270	Post path speed (U/seconds)	0.823	Initial path speed (U/seconds)	0.212	0.99	0.974	1.006	Post path speed (U/seconds)	0.688	1.003	0.989	1.017

Using the variables out of the equation method, none of the variables are significant predictors of pregnancy. However, when the variable in the equation method was used several discriminators of pregnancy did occur. These included initial concentration and initial concentration motile, post-processing concentration motile, and post processing total motile sperm count. Pre-processing total motile sperm count was not a predictor of pregnancy in the variable in or out of the equation models. M; Million, OR; Odds ration, CI; Confidence interval, [a]; <0.05 statistically significant.

Next, stepwise logistic regression analysis was performed (Table 4). Using variables out of the equation method, none of the variables are significant predictors of pregnancy. However, when the variable in the equation method was used several discriminators of pregnancy did occur. However, pre-processing TMSC was not one of these discriminators. Post-processing TMSC remained a significant predictor of pregnancy with this modality (P=0.009). To further evaluate whether TMSC was not a significant predictor of results because it depends on sperm volume, concentration, and motility, the analysis was repeated without these three parameters. However, pre-processing TMSC failed to reach significance in this analysis P=0.653, odds ratio (OR)=1.001, confidence interval (CI)=0.999 to 1.002

Discussion

Many studies have found the pre-processing and post-processing TMSC to be a significant predictor of pregnancy, while other studies have not detected a difference (3-8, 11). The results of these studies have found that a TMSC between 1 million and 20 million is a cut-off for pregnancy after IUI (3, 4, 6-8). This discrepancy in TMSC cut-off required investigation. The results of this study suggest that any difference detected may be the result of confounding effects between sperm parameters. With this study only 1 out of 3 methods used to predict pregnancy found TMSC to be a significant predictor of pregnancy, and this was only considering post-processing analyses. If a statistical test such as the student's t test is performed significant differences are found, which may become insignificant when controlling for confounding effects between semen analysis results. It could be hypothesized that based on the variable in the equation method of stepwise logistic regression some variables do predict pregnancy outcomes. In that case, only post-processing semen analysis results should be considered significant. It should be noted that the post-processing TMSC with the variable out of the equation method of stepwise logistic regression analysis trended towards being a significant predictor of pregnancy. It may remain a significant predictor of pregnancy in a larger study, although with 2231 IUIs this study is robust. (This can be confirmed by the small CI generated with the logistic regression analysis). Hamilton et al. (3) argued that a semen analysis is only valid if it is correlated with pregnancy and not with other factors, which supports our use of pregnancy as the predictor. It should be considered, that since certainly pre-processing TMSC and likely post-processing TMSC only can predict pregnancy due to confounding effects, we have likely found the explanation of why the predictive cut-off for TMSC varies so substantially in different studies and why certain studies have failed to detect TMSC as a predictor of pregnancy outcomes at IUI (3-11).

When running a logistic regression (which predicts the correlation of the sperm parameters being studied to pregnancy outcome) the variable in the method established no correlations, and as such no significant results. The variable out method established a significant correlation between post-processing TMSC and pregnancy outcome. A possible explanation for the different outcomes of the two logistic regression analyses can be explained by the variable selection problem. The variable selection

problem explains that when computation of the linear regressions occurs, the computer determines which variable to add into the equation (variable in method), or which variable to take out (variable out method) to compute the smallest P value possible (13). Therefore, the variable in the method begins with no variables and adds a variable one by one, computing a P value for each variable when added into the equation. On the other hand, different results were calculated by beginning with all the variables and removing one variable at a time, again computing a P value for each variable. These variables received different P values in each method since the variable was added or removed, by the program computing the logarithmic regression. Therefore, the same variable generated different p values based on when it was added or removed from the equation. It should be noted that either the variable out of the equation or the variable in the equation method are acceptable tests to generate prediction models. It is up to the researcher a priori to determine which will be used.

The strengths of this study include the prospective nature and inclusion of a moderately robust patient population. The weaknesses of this study include that: semen analyses are well known for variations between tests, and the use of the CASA also has important inherent limitations- such as the inability to obtain accurate counts and percent motilities when the concentration of specimens is very high or quite low, or when a specimen is contaminated with debris. Another weakness is that this study does not contain data on 100,000 or more insemination cycles. However, if that had been done, we may have been able to generate statistical significance with all variables, which would not have represented a true clinical significance, a risk with ultra-large data.

Conclusion

This study suggests that the value of TMSC in predicting pregnancy may be due to confounding effects. This would imply that as other parameters change in the semen analysis, the total motile sperm count may lose its significance and legitimacy as a predictor of pregnancy. This finding of confounding effects may explain the diverse cut-off values of TMSC as a predictor of pregnancy in the medical literature ranging from 1 million to 20 million depending on the study reviewed or the failure to detect TMSC as a predictor of pregnancy in other studies. If any parameters do predict pregnancy, they are the post-processing results. In conclusion, at the time of insemination, based on the semen parameters, it is unlikely that a physician can adequately counsel a couple on the likelihood of success.

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Authors' Contributions

M.H.D.; Was involved in the data collection. M.H.D., T.F.I.; Were involved in the planning of the analysis, the analysis of the data and wrote the initial manuscript. M.H.D., T.F.I., S.N., S.-L.T.; Were involved in the interpretation of the data. S.-L.T., S.N.; Were involved in the editing of the manuscript. All authors approved of the final version of this manuscript.

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