Sperm DNA fragmentation and mitochondrial membrane potential combined are better for predicting natural conception than standard sperm parameters

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Objective: To evaluate whether DNA fragmentation and/or mitochondrial membrane potential (MMP) predict natural conception better than standard sperm parameters.
Design: Prospective cross-sectional study.
Setting: University medical center.
Patient(s): Eighty-five infertile and 51 fertile men.
Intervention(s): Assessment of sperm DNA fragmentation, MMP, and standard semen parameters over a 6- to 12-month observation period.
Main Outcome Measure(s): Comparison between the results of DNA fragmentation, MMP, and standard sperm parameters alone or combined and achievement of natural conception.
Result(s): Twenty-six of the 85 (31%) men from infertile couples conceived naturally. The median values of DNA fragmentation and MMP in the men who conceived within the observation period were similar to those in the fertile controls. Optimal threshold values of DNA fragmentation and MMP were 25% as determined by receiver operating characteristic analysis (area under the curve [AUC], 0.70; 95% confidence interval (CI) 0.58–0.82) and 62.5% (AUC, 0.68, 95% CI 0.56–0.80), respectively. The men in the infertile group with values of DNA fragmentation $\leq$25% and with MMP values $\geq$62.5% had significantly higher odds for conception (odds ratio [OR], 5.22; 95% CI 1.82–14.93] and OR, 4.67; 95% CI 1.74–12.5, respectively). Normal semen analysis alone had no predictive value for natural conception (OR, 1.84; 95% CI 0.67–5.07]). Both sperm function tests combined had significant odds for natural conception (OR, 8.24; 95% CI 2.91–23.33]), with a probability of 0.607 (60.7%) for both normal values and 0.158 (15.8%) for abnormal values.
Conclusion(s): Sperm DNA fragmentation and MMP combined may be superior to standard semen parameters for the prediction of natural conception. (Fertil Steril® 2016;105:637–44. ©2016 by American Society for Reproductive Medicine.)
Key Words: Male infertility, sperm DNA fragmentation, sperm mitochondrial membrane potential, semen analysis, natural conception

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According to the World Health Organization (WHO), infertility is defined as the absence of conception after 1 year or more of regular unprotected sexual intercourse (1). Up to 14% of couples in industrialized countries are considered to be infertile, with the distribution of infertility due to male factor ranging from 20% to 70% (2, 3). Eighty percent of couples achieve pregnancy after 1 year; the remaining 20% (also considered fertile) will require 1 year more. However, in practice, after 1 year of unsuccessful attempts to conceive naturally, couples are referred for further clinical assessment (4).

Semen analysis alone provides important information on male fertility

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(5, 6). It is most useful in the diagnosis of extreme male factor infertility such as azoospermia, nonmotile sperm, and teratozoospermia (7) but is less reliable in distinguishing between fertile and subfertile men, that is, in identifying those couples who are likely to conceive without medical assistance and those who are not (8, 9), although clinical prediction models of natural conception, including standard sperm parameters, have been designed (10–12). As an extension to standard semen analysis, sperm function tests have been developed (13). As the inherent sperm DNA and mitochondrial integrity of the male gamete are a prerequisite for a successful conception, they are supposed to predict the male fertility potential (14). Samples with low proportions of sperm with DNA fragmentation (15) and high mitochondrial membrane potential (MMP) are highly indicative of normal fertility potential (16).

Most of the available techniques for the detection of sperm DNA integrity provide limited information on the nature of the DNA lesions detected, and none of them can fully depict the exact etiology and pathogenesis of impairment of sperm DNA integrity (17). The deoxynucleotidyl transferase-mediated fluorescein nick-end labeling (TUNEL) assay is one valuable tool in the evaluation of DNA integrity (17, 18). Impaired DNA integrity is associated with prolonged time to pregnancy, a very low probability of achieving a natural conception (15, 19) or conception after IUI (20), and increased pregnancy loss (21). Data on a relationship between DNA sperm fragmentation, measured by TUNEL, and natural conception are scarce and inconsistent (22, 23).

Mitochondria are multifunctional organelles that play an important role in cell function, and regulated interactions between nuclear and mitochondrial DNA are required to ensure mitochondrial biogenesis (24). The peculiarity of highly regulated spermatogenesis with rapid turnover of sperm germinal epithelium is that they possess different mitochondria, which are a condition for a high sperm fertilizing ability (25, 26).

Sperm MMP provides useful information about a man's fertility potential, reflects sperm quality, and can be assessed by specific fluorescent markers (16, 26–29); low sperm MMP reflects cells of low quality, which are associated with reduced conception rates (16).

The aim of this study was to evaluate [1] whether sperm DNA fragmentation (assessed by TUNEL), MMP (measured by means of DiOC6(3)), and semen analysis based on WHO criteria and on the threshold values we established within the observation period may have a predictive value on natural conception by infertile couples and [2] whether these tests may be useful in distinguishing between couples who have good chances for natural conception and those requiring assisted conception.

PATIENTS AND METHODS

Study Population

This 3-year prospective, cross-sectional, observational study was carried out from 2010 to 2013 at the outpatient infertility clinic, Andrology Unit, Department of Obstetrics and Gynecology of the University Medical Centre Ljubljana. The study was approved by the national medical ethics committee (consent number 110/06/10).

Overall, we consecutively enrolled 136 nonazoospermic men: 85 infertile and 51 fertile men. The infertile men from infertile couples presenting for fertility evaluation with a 12- to 18-month history of unsuccessful attempts to conceive were observed for an additional 6–12 months, with the overall time of unprotected regular sexual intercourse thus amounting to 24 months (infertile group, group I); their female partners had verified tubal patency and normal FSH levels (below 11 IU/L), were endometriosis free, and were younger than 37 years. The fertile men were partners of currently pregnant women who had conceived within the previous year (fertile group, group II). They were recruited at the department's outpatient clinics during the mother's pregnancy examination and had no history of infertility. Before enrollment, each couple gave an informed consent for participation in the study. At the end of the observation period, group I was divided into two groups based on the occurrence of conception. The subgroup of men who did not conceive was called group IA, and the subgroup of men who conceived within the observation period was called group IB. Natural conception prediction was defined as conception prediction.

Standard Sperm Analyses

Semen samples were provided by masturbation after 2–5 days of sexual abstinence. Semen was assessed according to WHO guidelines (13). To evaluate sperm morphology, the strict criteria were used. The men with normal values of all three parameters were classified as normozoospermic (normal semen analysis) when sperm concentration was $\geq 15 \times 10^6$/mL, progressive motility (motility) was $\geq 32\%$, and normal morphology $\geq 4\%$ (all above the 5th percentile of the lower reference value proposed by WHO guidelines) (13) or when all were above the threshold values we statistically calculated (receiver operating characteristic [ROC] analysis) on the basis of the variables obtained in the infertile group (group I).

Semen preparation for flow cytometry (FCM) of sperm, DNA fragmentation, and MMP semen (volume 1.5 mL) was layered on a two-step discontinuous PureSperm (Nidacon, International AB) concentration gradient (100%/40%) and centrifuged at 160 *g* for 30 minutes. Then the sperm pellet from the 100% layer was washed with 2 mL of SpermPrep medium (MediCult), centrifuged at 220 *g* for 10 minutes, and resuspended in 0.5 mL of SpermPrep medium. An aliquot of prepared sperm at a final concentration of 1×10^6/mL was used for marker detection.

MMP was measured by means of 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3) staining; Molecular Probes Eugeon, Oregon, USA). Propidium iodide (PI) was used as supravital fluorescent stain. Sperm were incubated in 1 mL of 0.5 nmol/L DiOC6(3) dye at 37°C in a CO_2 incubator for 20 minutes. DiOC6(3) has a single wave length emission; a normal MMP was attributed to the cells with high fluorescence signals.

Sperm DNA fragmentation was evaluated using a TUNEL assay (In Situ Cell Detection Kit, Fluorescein, Roche). The concentration of fresh semen was adjusted to 2×10^7 cells/mL.

The semen suspension was fixed in 2% formaldehyde in phosphate buffered saline (PBS) and staining solution (0.1% Triton X-100 v 0.1% sodium citrate) for 1 hour. After the second wash in PBS, the sperm pellets were resuspended in 50 mL of freshly prepared staining solution for 60 minutes. All fluorescence signals of labeled sperm were analyzed by the flow cytometer FACSCanto (Becton Dickinson); 30,000 sperm were examined for each assay at a flow rate of about 100 cells/s (18).

Statistical Analysis

The analysis was performed using SPSS 15.0. Distribution of median values of all parameters between infertile (divided in two groups as described above) and fertile men were tested using the Kruskal-Wallis test or χ^2-test, as appropriate. The Mann-Whitney test was used for pairwise multiple comparisons using Benjamini Hochberg adjustment. Pearson correlation coefficients were calculated for DNA fragmentation and MMP and sperm concentration, motility, and morphology. Logistic regression analyses were used to test the association among standard sperm parameters, DNA fragmentation, and MMP. ROC analysis was performed to determine the threshold values of standard semen parameters and sperm function tests. Univariate and multiple logistic regressions were used to test the effect of normal semen analysis and of sperm function tests on natural conception. Normal semen analysis was defined with three parameters as described above. The sperm function tests were combined into the test parameter if both values were normal according to the threshold values determined by ROC analysis. The results of all groups are expressed as medians (range) or numbers (%), unless otherwise stated. $P<.05$ was considered statistically significant.

RESULTS

Clinical and Sperm Characteristics of 136 Infertile and Fertile Men

Within the observation period, 31% (26/85) of infertile couples (group I) conceived and delivered after 37 weeks of pregnancy (group IB), whereas 69% (59/85) did not conceive spontaneously (group IA). On inclusion, the median (range) duration of infertility in infertile men (n = 85) was 18 (8–20) months. Those who conceived (group IB, n = 26) were observed for an additional median time (range) of 9 (4–16) months, and those who did not (group IA, n = 59) for 6 (4–12) additional months.

Patients' clinical characteristics, standard sperm parameters, DNA fragmentation, and MMP are reported in Table 1.

There was no statistically significant difference in the presence of a varicocele, history of orchidopexy, and female age between infertile (groups IA and IB) and fertile groups (group II); the differences reached statistical significance in male age, standard semen parameters, and sperm function tests (Table 1). The male median age (range) was statistically significantly different between the men who did not conceive (31 years, range 20–42 years; group IA) and fertile men (33 years, range 25–47 years; group II; $P=.002$), whereas no statistically significant difference between any other groups was found (IA vs. IB and II vs. IB; $P>.05$; Table 1; data not shown in Fig. 1). The highest median values (range) of standard sperm parameters were found in fertile men (group II): concentration 95 $\times$ 10^6/mL (2−230 $\times$ 10^6/mL); motility 55% (30%–75%); morphology 35% (7%–59%), and all pairwise differences between this group and groups IA and IB were statistically significant (all $P<.001$; Table 1). The lowest median values (range) were found in the men who did not conceive (group IA): 28 $\times$ 10^6/mL (0.4–213 $\times$ 10^6/mL), 45% (5%–60%), and 12% (1%–55%), respectively (Table 1, Fig. 1). In the men who conceived (group IB), the values were the following: 54.5 $\times$ 10^6/mL (6−134 $\times$ 10^6/mL), 50% (20%–65%), and 15% (3%–45%), respectively. The only statistically significant difference between group IA and group IB was found in sperm motility ($P=.01$) but not in concentration ($P=.105$) or morphology ($P=.126$; Fig. 1). Median sperm DNA fragmentation (range) was significantly higher in group IA (31%, 0%–88%) than in group II (15.5%, 1%–73%; $P<.001$) and group IB (18%, 3%–55%; $P=.008$). The values between the groups II and IB were very similar ($P=.547$). A reverse but similar situation was observed in MMP values. MMP was significantly lower in group IA (50%, 11%–94%; $P<.001$) versus group II (74%, 20%–97%; $P=.013$) versus group IB (65.5%, 28–94%), but similar in the fertile men and the men who conceived ($P=.198$; Table 1, Fig. 1).

Relationships Between DNA Fragmentation and MMP and Standard Sperm Parameters

A statistically significant correlation was found between the two function tests and standard semen parameters ($P<.001$). Sperm DNA fragmentation and MMP were negatively correlated ($r = -0.69$; $P<.001$). Sperm motility was negatively associated with DNA fragmentation ($r = -0.45$; $P<.001$). Both sperm morphology and motility were positively associated with MMP ($r = 0.57$, $P<.001$; $r = 0.66$, $P<.001$). After performing the multiple logistic regression that was used to test the association between a single sperm function test and normal standard semen analysis (5th centile, WHO), only MMP was positively associated with normal semen analysis (OR, 1.9; 95% CI 1.34–2.71; data not shown).

Predictive Threshold Values of Sperm Function Tests and Standard Sperm Parameters

A discriminating power with optimal threshold values and test performance results (Table 2) were determined for each semen assessment method for the infertile group (group I).

The odds (OR, 5.22; 95% CI 1.82–14.93) for optimal DNA fragmentation threshold values (≤25%) were statistically significantly higher in the group who conceived (20/26 [77%]) in comparison with the group who did not (23/59; 39%). In 17 of the 26 (65.4%) men who conceived (group IB), the MMP value was above the threshold (≥62.50%), with higher odds for conception (OR, 4.67; 95% CI 1.74–12.5) in comparison with the men who did not conceive (group IA), where 17 of the 59 (28.8%) had normal values. In 15 of the 26 (57.7%) men who conceived (group IB), the

TABLE 1

Patients' clinical characteristics, standard semen parameters, DNA fragmentation, and MMP by groups.

Characteristic	Infertile group (group I) (n = 85)	Fertile group (group II) (n = 51)	Subgroup of men who did not conceived within observation period (group IA) (n = 59)	Subgroup of men who conceived within observation period (group IB) (n = 26)	*P* value
Male age (y)	31 (25–43)	33 (25–47)	31 (20–42)	32.5 (25–43)	.005[a]
Female age (y)	30 (20–36)	31 (24–40)	29 (22–36)	30 (20–35)	.085[a]
Varicocele	14 (16.5)	3 (5.9)	11 (18.6)	2 (7.7)	.167[b]
Orchidopexy	7 (8.2)	2 (3.9)	3 (5.1)	4 (15.4)	.136[b]
Standard sperm parameters					
Sperm concentration ($\times 10^6$/mL)	31 (0.4–213)	95 (2–230)	28 (0.4–213)	54.5 (6–134)	<.001[a]
Progressive sperm motility (%)	45 (5–65)	55 (30–75)	45 (5–60)	50 (20–65)	<.001[a]
Sperm morphology (%)	13 (1–55)	35 (7–59)	12 (1–55)	15 (3–45)	<.001[a]
Sperm function tests					
DNA fragmentation (%)	24 (0–88)	15.5 (1–73)	31 (0–88)	18 (3–55)	<.001[a]
MMP ($\Delta\psi$m) (%)	52 (11–94)	74 (20–97)	50 (11–94)	65.5 (28–94)	<.001[a]

Note: Values represent median (range) or n (%); *P* values for the comparison of the fertile group (group II), the subgroup of men who did not conceived within the observation period (group IA), and the subgroup of men who conceived within observation period (group IB) are shown.
[a] Kruskal-Wallis test.
[b] Chi-square test.

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values for motility were above the determined threshold value (≥47.5%), with an OR of 3.03 (95% CI 1.16–7.89) in comparison with 18 of the 58 (31.0%) men who did not conceive. The optimal threshold value for sperm concentration (≥63.5 × 10^6/mL) was found in 12 of the 26 (46.2%) men who conceived (group IB), with an OR of 3.74 (95% CI 1.36–10.29) in comparison with the men who did not conceive (group IA), where 11 of the 59 (18.6%) men had a normal value. The odds (OR, 2.63; 95% CI 0.99–7.01) for an optimal threshold value for morphology (≥18%) was observed in 12 of the 26 (46.2%) men who conceived (group IB) in comparison with 14 of the 57 (24.6%) men who did not conceive (group IA; Table 2, Supplemental Fig. 1).

Association Among Sperm Function Tests, Semen Analysis, and Ability to Conceive

The effect of the normal levels of the two function tests was highly significant in itself (Table 3) or when combined with normal semen analysis ($P<.001$; models I and II), whereas the normal semen analysis alone (Table 3) based on WHO criteria (model I) or on the threshold values we determined (model II) was not associated with conception ability ($P>.05$). The men with normal results of the combined function tests had significantly higher odds for conception in comparison with those with nonnormal results of the combined sperm function tests (OR, 8.24; 95% CI 2.91–23.33), with a calculated probability of successful conception of 0.61 (60.7%) for the men with normal values (17 men [65.4%] in group IB and 11 men [18.6%] in group IA) and 0.158 (15.8%) for those with abnormal ones (n = 9 men [34.6%] in group IB and 48 [81.4%] in group IA). Using the values of combined sperm function tests values as a discriminating factor, 77% of men were classified correctly (sensitivity [Se] = 0.65; specificity [Sp] = 0.81; positive predictive value [PPV] = 0.61; negative predictive value [NPV] = 0.84; positive likelihood ratio [LR+] = 3.5; negative likelihood ratio [LR-] = 0.43; Table 3).

All relevant predictive values of the observed parameters are presented in Supplemental Figure 1.

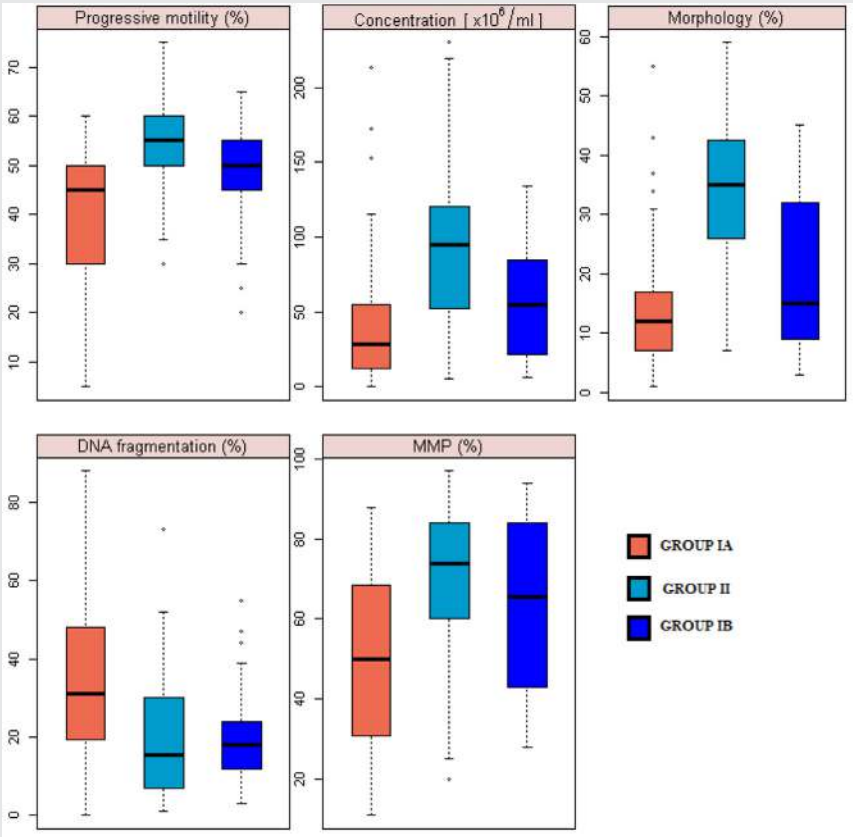
DISCUSSION

The results of our study suggest that male fertility potential leading to natural conception and further to live birth may be better predicted by the combination of DNA fragmentation and MMP simultaneous testing than with standard semen analysis alone. To our knowledge this is the first study combining the results of simultaneously measured sperm function tests with the threshold values we calculated for each study subject with the aim of better predicting conception.

Our results on different median values of DNA fragmentation and MMP in fertile versus infertile men are in agreement with previous reports (16, 30, 31). In addition, correlations between DNA fragmentation, MMP, and standard sperm parameters confirm the previous findings (25, 27, 32, 33). However, the positive association between MMP and motile and morphologically normal sperm forms and the negative association between motility and DNA fragmentation observed in our analysis are likely to be important since sperm motility may depend on the functional integrity of mitochondria and represent cells with lower chromatin damage, which is also expressed through normal sperm morphology as confirmed previously (25, 27, 34). Moreover, the association between normal semen analysis (above the 5th centile, WHO guidelines) and MMP further confirms the importance of sperm cell mitochondrial integrity.

The evidence regarding the intactness of the inner mitochondrial membrane and its role in proper sperm motility and good fertilizing ability is beginning to accumulate

FIGURE 1



Note:

Subgroup of man who did not conceived within observation period (**Group IA**/n=59)

Fertile group (**Group II**/n=51)

Subgroup of men who conceived within observation period (**Group IB**/n=26)

Box plots present distribution of standard semen parameters, sperm DNA fragmentation and mitochondrial membrane potential (MMP) in the three groups.

Comparison of three groups achieving natural conception.

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(25, 27, 29, 33). During the final step of sperm differentiation, most of the cytoplasm and mitochondria are lost, and the remaining amount is retained in the midpiece (35). Another matter of debate is the possible degeneration of mitochondria inside the oocyte cytoplasm within 1–2 days and the crucial role of mitochondria in the fertilizing ability of sperm more than in further embryo development (36, 37). Thus, the functionality of sperm mitochondria might better influence fertilizing ability than embryo quality (16). Our findings demonstrate negative effects of MMP disruption on the achievements of conception in infertile men who did not conceive within 24 months of regular unprotected sexual intercourse. The minimal threshold value of 64% for sufficient MMP has been shown to be connected with unsuccessful attempts to achieve assisted conception (16). The results we obtained are in agreement with this previous report. However, as the impact of MMP values has never been confirmed on the population of fertile men, it is noteworthy that we have found MMP to be superior to standard semen analysis in providing information on sperm function as well as the predictive value for conception.

DNA integrity damages can be due to intrinsic factors like deficiencies in natural processes such as chromatin packaging and abortive apoptosis or to extrinsic factors (32, 38–41). MMP has been associated with elevated levels of reactive oxygen species (31). Furthermore, susceptibility of sperm to oxidative stress through changed mitochondrial membrane

TABLE 2

Predictive properties of sperm DNA fragmentation, MMP, and standard semen parameters values for natural conception by ROC analysis.

Threshold value	AUC (95% CI)	Se (95% CI)	Sp (95% CI)	PPV	NPV	OR (95% CI)	LR (+)	LR (−)
Sperm DNA fragmentation (≤25%)	0.70 (0.58–0.82)	0.77 (0.58–0.92)	0.61 (0.49–0.75)	0.47	0.86	5.22 (1.82–14.93)	1.97	0.38
MMP (Δψm) (≥62.5%)	0.68 (0.56–0.80)	0.65 (0.46–0.85)	0.71 (0.59–0.83)	0.50	0.82	4.67 (1.74–12.50)	2.27	0.49
Sperm concentration ($\geq 63.5 \times 10^6$/mL)	0.62 (0.49–0.75)	0.46 (0.27–0.65)	0.81 (0.71–0.92)	0.52	0.77	3.74 (1.36–10.29)	2.48	0.66
Progressive sperm motility (≥47.5%)	0.68 (0.56–0.81)	0.58 (0.39–0.77)	0.69 (0.58–0.81)	0.46	0.78	3.03 (1.16–7.89)	1.86	0.61
Sperm morphology (≥18%)	0.61 (0.48–0.74)	0.46 (0.27–0.65)	0.75 (0.63–0.86)	0.46	0.75	2.63 (0.99–7.01)	1.88	0.71

Note: All values acquired from the infertile group (group I; n = 85). AUC = area under the curve.

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permeability with possible subsequent MMP loss, production of reactive oxygen species, and DNA fragmentation has also been confirmed on sperm cells (42). This interconnection might not be the only major impact on sperm DNA fragmentation (43). Nevertheless, if the damage to the sperm is low, the oocyte can use its repair mechanisms and preserve the development of the embryo (37).

The threshold values of DNA fragmentation, measured by TUNEL, vary from 4% (44) to 36.5% (45), the wide variation being due to different laboratory protocols for the method (46). Our results are comparable to the threshold values of 19.25% (47), 20% (48), and 26% (49), as measured by TUNEL. Although these threshold values were determined using the ROC analysis, the discriminative power was connected with assisted conception. We used TUNEL without PI coupled with FCM to assess DNA integrity. TUNEL assay has been shown to be accurate, objective, precise, and reproducible in detecting the true extent of DNA damage (47, 48, 50) as well as comparable to the discriminative and predictive values of other methods (51). We presume that the method's reliability is also due to the high number of cells (30,000) examined. In addition, FCM takes only a few hours in comparison with standard semen analysis.

The men in our study with the proportion of intact sperm cells at or below the defined cutoff values for DNA fragmentation and at or above MMP cutoff values had approximately five-fold greater odds for natural conception, with a NPV of more than 80%, thus confirming that semen samples with low proportions of sperm DNA fragmentation (15, 52) and high MMP are indicative of fertility potential (16).

In addition, the median values of both sperm function tests among the men who conceived in our study were close to the values in men of proven fertility, whereas the standard semen analysis composed of three main parameters with the lowest WHO criteria (5th centile) was not discriminative. Furthermore, although the results of ROC analysis are supposed to be most reliable, the obtained values of sperm concentration and morphology, but not motility, classified the men as infertile, which is partially in line with previous studies (5, 53).

Our results indicate that sperm cell function measured simultaneously by DNA fragmentation and MMP is positively correlated to natural conception. Those men with normal values on both tests had an eight times greater odds of conceiving naturally, whereas 80% of men with abnormal values on both tests were correctly classified as infertile. Thus, it is more appropriate to diagnose infertile males falsely as fertile instead of diagnosing fertile males as infertile. While this finding is helpful, it is not conclusive and should be validated on a larger independent sample.

Although studied more intensively than MMP, sperm DNA integrity remains controversial and even confusing depending on the detection method used. Uncontrolled confounding, heterogeneity, and a potential selection bias are

TABLE 3

Regression model including normal sperm analysis and combined sperm function tests.

	Univariate		Model I		Model II	
Variable	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Normal semen analysis (5th centile WHO)[a]	1.84 (0.67–5.07)	.241	0.74 (0.21–2.57)	.632		
Normal semen analysis (infertile men)[b]	3.13 (0.93–10.51)	.065			1.25 (0.31–5.11)	.752
Normal function tests[c]	8.24 (2.91–23.33)	<.001	8.96 (2.75–29.18)	<.001	7.39 (2.42–22.56)	<.001

[a] Concentration, $\geq 15 \times 10^6$/mL; progressive motility, ≥32%; morphology, ≥4%.
[b] Infertile group (n = 85); concentration, $\geq 63.5 \times 10^6$/mL; progressive motility, ≥47.5%; morphology, ≥18%.
[c] DNA fragmentation (≤25%) and MMP (≥62.5%).

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of concern in the majority of evidence and are the reasons for the disparity of results (23). Consequently, our findings may be compared only partially to other studies, and with caution. As stated in the recent guidelines of the American Society for Reproductive Medicine, the clinical utility will only be confirmed after standardization of methodology and clinically applicable threshold values, which would be reached by the same criteria (23).

Our results also demonstrate some uncertainties as well as false detection rates, which were expected considering the small sample size; still unknown sources of variability connected to unique sperm cell biology; and lack of appropriate data validation. We consider this to be one of our study limitations.

To reduce the effect of possible confounding factors on conception we enrolled only the couples with no female pathology present, which is one of the strongest aspects of our evidence. In addition, a very homogenous male population was studied, and a potential impact of other male pathology was diminished as well.

We believe that our novel model might be of important clinical usefulness for clearly defined infertile groups of men such as ours. Irrespective of the results of standard semen analysis, these men will benefit from the combined sperm function tests in clinical decision making and hence might avoid unsuccessful attempts to conceive. Such decision making is time and cost-effective and has positive effects on the psychological aspect of infertility management.

To conclude, our results suggest that DNA fragmentation and MMP combined in the evaluation of infertile males may serve as good predictors of natural conception irrespective of results of standard semen analysis. Therefore, we propose they be considered in the management of male factor infertility.

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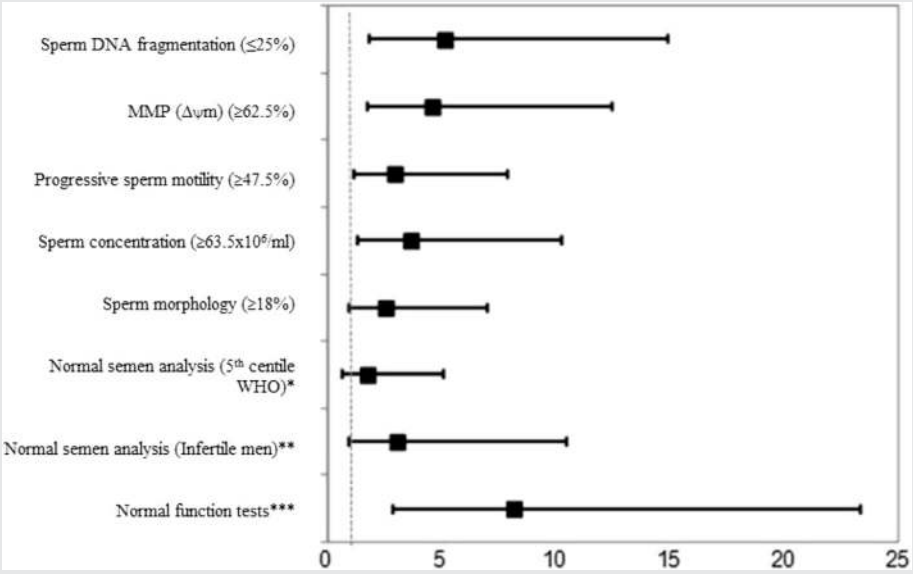
SUPPLEMENTAL FIGURE 1



Odds ratios with 95% confidence intervals for all relevant clinical tested parameters. * Sperm concentration $\geq 15x10^6$/ml; progressive sperm motility $\geq 32\%$; sperm morphology $\geq 4\%$. ** Sperm concentration ($\geq 63.5x10^6$/ml); progressive sperm motility $\geq 47.5\%$, sperm morphology $\geq 18\%$. *** Sperm DNA fragmentation ($\leq 25\%$) and sperm MMP – mitochondrial membrane potential ($\geq 62.5\%$). All values acquired from Infertile group - Group I (n=85).

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