

The Effects of Short Abstinence Time on Sperm Motility, Morphology and DNA mDamage

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Abstract

This was an experimental study to determine the influence of short abstinence time on sperm motility, morphology and DNA damage performed at the Assisted Reproductive Technology (ART) unit, Ramathibodi Hospital. Fifty-seven semen samples with normal semen analysis according to World Health Organization (WHO) 1999 and Kruger strict criteria were obtained. Volunteers were instructed to abstain for 3-5 days, 18-30 hours and 4 days for the 1st, 2nd and 3rd semen collection. Total motile sperm count (TMSC) was determined using computer-assisted semen analysis (CASA); sperm morphology was determined using eosin-methylene blue staining and DNA damage was assessed by TUNEL assay. The results showed that TMSC was not significantly different between the 1st (3-5 days abstinence time) and 2nd semen collection (18-30 hours abstinence time after the 1st collection) (p value=0.289). The percentage of DNA damage (4.6% vs. 9.8%, p value<0.001) and normal morphological sperm (14.9% vs. 17.2%, p value<0.001) in the 2nd semen collection were significantly lower than the 1st collection, although the value was still in the normal range. Semen parameters of the 3rd collection (4 days abstinence time after the 2nd collection) correlate with those of the 1st collection. We conclude that in normal semen, with an abstinence time of only one day, TMSC was not significantly different between 3-5 days abstinence time and 18-30 hours abstinence time. The result of DNA damage and the percentage of normal morphological sperm were significantly lower in 18-30 hours abstinence time.

Keywords: Total motile sperm count; World health organization; Semen; DNA

Introduction

Male infertility has been shown to represent 30-40% of the total causes of infertility in couples [1]. The examination of semen following World Health Organization (WHO) guidelines is important and remains the initial screen in the study of male infertility and for the evaluation of spermatogenesis and also has served to provide a standardized approach for the assessment of the fertility potential of semen samples for more than 25 years [2-5]. Therefore the result is used as a guideline for additional examinations and for further treatment of male infertility with regard to determining the preparation period before examination of semen, the WHO guidelines have determined to have the abstinence time before the examination be 2-7 days in order for the concentration of semen, percentage of motility of sperm and percentage of morphology of sperm to be within normal criteria. However, the basis for this recommendation is unclear because no supporting references are provided [5].

Generally, semen quality is affected by several factors such as abstinence duration, frequency of ejaculation, febrile illness, general health, infection of the genital tract, urogenital surgery and the environment [6-13]. With regard to abstinence time, there are only a few researchers studying this factor so no clear tendency has yet been discovered as to how many days are optimal to obtain the best results for semen parameters [8-10,12,13]. In this area studies are often retrospective; only a few have been prospective studies several semen parameters have been evaluated as predictors of pregnancy. It seems that the most important parameter to predict pregnancy rate is the Total Motile Sperm Count (TMSC) [14-16].

In addition there have been studies showing that the sperm DNA may be a better identifying factor as to the quality of sperm, apoptosis, or programmed cell death due to DNA damage. Recent studies have demonstrated an association between the integrity of sperm DNA

and pregnancy outcomes [17-19]. Practically, it has been found that sometimes a patient coming to check his semen did not have an abstinence time in accord with the specified standard because it was not convenient for the patient to check during the specified abstinence time, with collection being fewer or more days. In this study we evaluated the short abstinence time of only one day, with regard to the values of concentration of semen, the percentage of motility of sperm and the percentage of varying morphology of sperm as well as the DNA damage as compared to a collection time within the specified WHO guidelines.

Materials and Methods

The study protocol was approved by the Ethical Clearance Committee on Human Rights Related to Researches involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University. All volunteers were informed and signed informed consent forms.

Volunteers

66 healthy men between 18-44 years of age with 3-5 days abstinence were recruited in this study. A total of 57 healthy men have a normal semen analysis that was included in our study.

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Semen collection

For the 1st collection, 3-5 days abstinence was advised prior to semen collection for the 2nd collection; the volunteers were instructed to abstain 18-30 hours after the 1st collection. Finally, volunteers were instructed to abstain 4 days after the 2nd collection. All semen samples were obtained by masturbation into a wide mouthed plastic container in a separate room near to the semen analysis laboratory.

Semen analysis

Semen parameters were then evaluated after liquefaction at 37°C for 30-60 minutes, a routine semen analysis was performed using Computer Assisted Semen Analysis (CASA) according to the World Health Organization 1999 guidelines to determine concentration and motility [5].

Sperm morphology

Sperm morphology was determined using by the strict criteria laid down by Kruger et al. [20]. After preparation of slides, sperm were stained using eosin and methylene blue. A total of 200 sperm were counted at 100X with an oil-immersion bright-field objective lens at 400X magnification.

DNA integrity assessment

DNA integrity was determined using an *in situ* Nick-end Labeling (TUNEL) detection kit (*In situ* Cell Death Detection Kit, POD; Roche, Mannheim, Germany) Semen was centrifuged at room temperature for 10 minutes at 1900 rpm. The supernatant was discarded, and the remaining pellet was washed twice in Phosphate-Buffered Saline (PBS), pH 7.4, (Invitrogen Corporation, Scotland, United Kingdom), and re-suspended in the PBSA droplet of the sperm suspension was smeared onto a pretreated glass slides (Microscope slide, China), air dried, and fixed by immersion in freshly prepared 4% paraformaldehyde in PBS, pH 7.4 for 1 hour at room temperature. Next, the slides were rinsed in PBS for 5 minutes at room temperature, treated with pre-chilled 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice, and rinsed twice with PBS for total of 5 minutes at room temperature. Excess liquid was removed by tapping the slides. Then, the staining was performed according to the manufacturer's instructions.

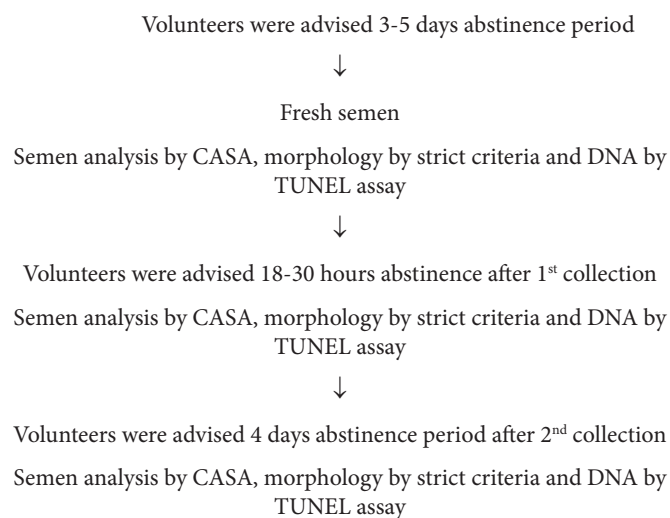
Visualization and evaluation of sperm DNA fragmentation was performed using a Nikon (Tokyo, Japan) photomicroscope equipped with epifluorescent light under appropriate filters. Sperm heads were examined at 1,000X magnification, and each was recorded as either green staining on the sperm head (positive, sperm with fragmented DNA), or red staining (negative, sperm without fragmented DNA) A total of 500 sperm was assessed from each subject, and the percentage of sperm cells with positive staining was calculated (Figure 1).

Statistical Analysis

Results were analyzed by using the program STATA® version 9 (Stata Corp, TX, USA). The data was presented as mean ± SD. Data with normal distribution were analyzed by the paired Student's *t*-test. Data without normal distribution were analyzed by using the Wilcoxon signed-ranks test *p*-value < 0.05 was considered as statistical significant.

Results

Protocol Flow Chart



Of the sixty-six men who volunteered, nine had abnormal semen analysis by WHO criteria; therefore fifty-seven volunteers completed the study. The mean (SD) age was 22.8 years (5.5), ranging from 18-44 years. The mean (SD) abstinence period in the 1st collection was 3.4 days (0.9), ranging from 2-5 days. We analyzed within-subject semen parameters and DNA damage variation between 1st, 2nd and 3rd semen collections.

Sperm volume and concentration of the 3rd collection were significantly higher than those of the 1st collection (*p* value 0.007, 0.009 respectively). There were no significant differences between TMSC, percentage of normal morphology and percentage of DNA damage when the 1st and the 3rd collections were compared (Table 1).

Sperm characteristics of the 2nd collection (18-30 hours abstinence time after the 1st collection) were significantly lower in volume, percentage of normal morphology and percentage of DNA damage than that of the 1st collection (*p* value < 0.001 in all). There were no significant differences between TMSC and sperm concentration when the 2nd and the 1st collections were compared (Table 2).

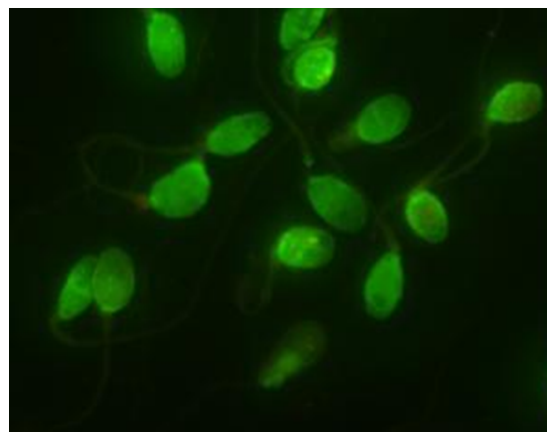


Figure 1: Positive control showed 100% of detection by TUNEL assay for spermatozoa which was damaged by adding DNase on sample.

Sperm characteristics of the 2nd collection showed a significantly lower volume, sperm concentration, percentage of normal morphology, and percentage of DNA damage than that of the 3rd collection (p value < 0.05 in all). There was no significant difference between TMSC when the 2nd and the 3rd collections were compared (Table 3 and Figure 2).

Discussion

A number of previous studies have investigated the influences of ejaculatory abstinence on semen parameters [8-10, 11-13, 20]. However, for many of those studies, conclusions were typically based on single ejaculate data for individuals within a population of men resulting in data with between-subject variation. Population studies provide meaningful information, although they fail to reveal variations in semen parameters within an individual over specified time frames. The present study represents one of only a few published reports that have evaluated the influence of defined periods of ejaculatory abstinence on within-subject semen parameters.

The previous studies reported that only 11 samples with short abstinence time showed significantly lower semen volume and concentration. Other semen parameters (pH, viability, total and grade motility and morphology) did not change [9]. Furthermore, a short abstinence time added immature chromatin in the spermatozoa. Our study is an experimental study, comparing semen parameters and DNA damage of the short abstinence period group in which abstinence time was 18-30 hours and the WHO recommended abstinence period group of 3-5 days. We decided to have an additional 3rd semen collection to increase the reliability of the 1st semen collection. We predicted that

if the 1st semen collection was a good control group, the results should correlate. From the results obtained from the 1st and the 3rd collection, the semen parameters indicated the same direction, so from the value of the semen parameters in the 1st semen collection it could be believed that volunteers' abstinence was as actually specified. We found that, with outcomes such as TMSC, the percentage of DNA damage and the percentage of normal morphological sperm, results were not different. There were differences in the volume and concentration; normally, regarding these two parameters, there is a lot of variation [21, 22]. There is a marked intra- and inter-individual variation in sperm concentration. For this reason it is usually recommended in clinical practice that more than one semen sample from an individual man be analyzed to get a reliable estimate of semen quality. In a previous study, which was published in the WHO 1999 laboratory manual for the examination of human semen and sperm-cervical mucus interaction guideline for sperm evaluation, this was shown as well [5].

Our results showed that there were no differences in the concentration and TMSC, which were the primary measured outcomes when comparing semen parameters of the 1st and the 2nd collections. There was the tendency for the 2nd collection to be better than the 1st collection. This result was comparable with the previous study reporting the effect of short or long abstinence periods in non-obstructive azoospermic patients indicating that there was low semen volume and concentration in the short abstinence period [13]. However, the volume, percentage of normal morphology and percentage of DNA damage were significantly lower in the 2nd collection when compared with the 1st collection, we also found that the values of all these parameters were still within normal range. This result may be explained by the creation of seminal fluid from the accessory gland being reduced. The percentages of DNA damage and normal morphological sperm were less because sperm was stored in epididymis for a short period [23]. We could not explain the reduction of the percentage of normal morphological sperm. This result might be influenced by the small sample sized. We also suggest to do more sample size in the further study. Previously it has been demonstrated that there was no change relative to abstinence between short abstinence period (1 day) and the percentage of DNA damage (tested by sperm chromatin structure assay) [9].

While our study found a reduction in the percentage of DNA damage (tested by TUNEL assay) in the short abstinence group, it was within normal range. The results of the study may be affected by the use of different tests to detect DNA damage [9].

Sperm Parameters	1 st Semen Sample	3 rd Semen Sample	p value
Volume	2.6(0.7)	2.9(0.6)	0.007*
Concentration (×10 ⁶ /ml.)	56.7(26.0)	66.1(31.1)	0.009*
Motility (%)	62.4(10.6)	60.8(14.2)	0.448
Total Motility (× 10 ⁶)	33.0(10-96.8)	37.5(6-107)	0.289
Normal Morphology (%)	17.2(3.7)	16.7(3.5)	0.294
DNA Damage (%)	9.8(4.5)	10.5(4.4)	0.231

*p value < 0.05

Table 1: Sperm parameters between 1st and 3rd collections.

Sperm Parameters	1 st Semen Sample	2 nd Semen Sample	p value
Volume	2.6(0.7)	1.9(0.6)	<0.001*
Concentration (× 10 ⁶ /ml)	56.7(26.0)	57.7(26.5)	0.744
Motility (%)	62.4(10.6)	65.3(14.3)	0.187
Total Motility (× 10 ⁶)	33.0(10-96.8)	39.0(3.9-84.5)	0.289
Normal Morphology (%)	17.2(3.7)	14.9(5.6)	<0.001*
DNA Damage (%)	9.8(4.5)	4.6(2.5)	<0.001*

*p value < 0.05

Table 2: Sperm parameters between 1st and 2nd collections.

Sperm Parameters	2 nd Semen Sample	3 rd Semen Sample	p value
Volume	1.9(0.6)	2.9(0.6)	<0.00*
Concentration (× 10 ⁶ /ml.)	57.7(26.5)	66.1(31.1)	0.043*
Motility (%)	65.3(14.3)	60.8(14.2)	0.075
Total Motility (× 10 ⁶)	39.0(3.9-84.5)	37.5(6-107)	0.597
Normal Morphology (%)	14.9(5.6)	16.7(3.5)	0.024*
DNA Damage (%)	4.6(2.5)	10.5(4.4)	<0.00*

*p value < 0.05

Table 3: Sperm parameters between 2nd and 3rd collections.

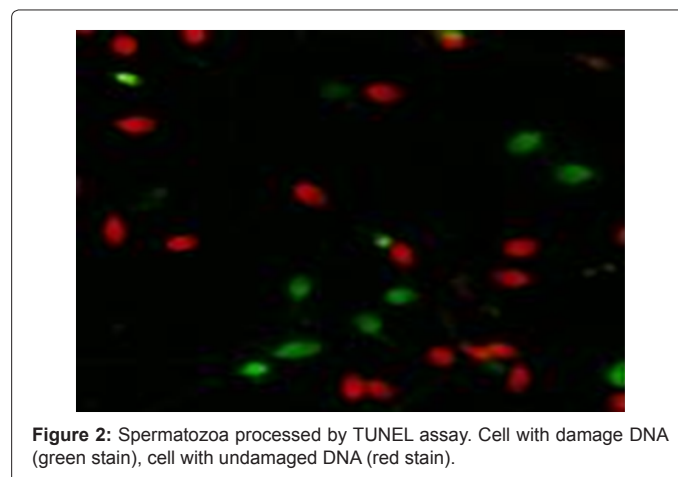


Figure 2: Spermatozoa processed by TUNEL assay. Cell with damage DNA (green stain), cell with undamaged DNA (red stain).

It was found that the etiology of sperm DNA damage appeared to be multi-factorial and might be due to either intrinsic or extrinsic factors. Various mechanisms can damage sperm DNA; abnormal chromatin packing, ROS and apoptosis are the most important etiological factors in disruption of DNA integrity [24-26]. The abstinence time was one of factors that might result in increasing or decreasing sperm DNA damage. Our study used an *in situ* TUNEL technique to assess sperm DNA damage because there has been shown to be a significant correlation between DNA denaturation *in situ* and the percentage of sperm labeling for DNA strand breaks. The TUNEL assay was used because it enabled a more precise assessment of the level of apoptosis of sperm [25]. A previous study had demonstrated that patients with high percentage of TUNEL-positive spermatozoa (>36.5%) showed a significantly lower mean pregnancy rate than those patients with a low percentage of TUNEL-positive sperm (<35.5%) [27]. Male infertility is associated with poor sperm DNA integrity. It has been suggested that abnormal DNA integrity may adversely affect fecundity in couples having natural sexual intercourse and in those treated by IUI, IVF and ICSI [17,26,28-30]. Yet in other studies, abnormal DNA integrity has been proposed as a cause of impaired late embryo development, but did not appear to be associated with poor fertilization because the paternal genome is transcriptionally inactive until two days after fertilization. Once the paternal genome is active it results in poor blastocyst development, implantation failure or early fetal loss [26]. Also DNA damage was inversely correlated with pro-nucleus formation [31,32].

The strength of this study is that the data obtained from the 1st and the 3rd collection moved in the same direction, so the control group was reliable when compared with the test group. Furthermore, the primary measured outcome, TMSC, of the control and test groups was not different, while the secondary measured outcome was different in a beneficial way, with the percentage of DNA damage and normal morphological sperm being less. Since this study was performed using normal semen, the results cannot be extrapolated to abnormal semen. Our data do not allow for estimation of an optimal duration of abstinence for fertility purposes. Further study is needed to confirm this parameter. This study is designed to detect the differences in the main variables of interest, TMSC, the percentage of normal morphological sperm and DNA damage, but these are the intermediate outcomes for infertility treatment and may not reflect the pregnancy outcomes.

Conclusion

We conclude that in normal semen there were no significant differences in terms of TMSC between 3-5 days abstinence time and 18-30 hours abstinence time (only one day of abstinence). However DNA damage and the percentage of normal morphological sperm were significantly lower in 18-30 hours abstinence time when compared with the 3-5 days abstinence time.

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