

## Short Abstinence May Have Paradoxical Effects On Sperms With Different Level Of DNA Integrity: A Prospective Study

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**Purpose:** To investigate the effect of short abstinence on sperm function tests and semen parameters.

**Materials and methods:** This prospective study included 65 male patients with increased DNA injury in their ejaculated sperm and a history of recurrent pregnancy loss and/or assisted reproductive techniques failures. The effects of antioxidants medical therapy and short abstinence on semen quality were assessed (TUNEL test and CMA3 staining).

**Results:** Antioxidants have statistically significant effects on mean sperm concentration (untreated,  $67.51 \pm 44.40$  million/ml, vs. treated,  $56.09 \pm 37.85$  million/ml;  $P$ -value=0.005) and mean TUNEL score (untreated,  $24.56\% \pm 9.49\%$ , vs. treated,  $20.64\% \pm 10.28\%$ ;  $P$ -value = 0.013).

Moreover, a short abstinence period might have positive effects as shown on the TUNEL assay ( $20.64\% \pm 10.28$  vs.  $17.38\% \pm 8.59$ ;  $P$ -value = 0.028) and CMA3 staining ( $47.79\% \pm 20.78$ , vs. short  $41.92\% \pm 18.49$ ;  $P$ -value = 0.019), when considering all study subjects. However, different results were obtained using more precise analysis based on a TUNEL cutoff score of 20%. The analysis showed that short abstinence might improve sperm DNA integrity in patients with TUNEL score > 20% (mean TUNEL score from  $27.85\% \pm 8.32\%$  to  $19.14\% \pm 8.90\%$ ;  $P$ -value = 0.001%). However, it might have deleterious effects on sperm DNA integrity in patients with TUNEL score < 20% (mean TUNEL score from  $11.89\% \pm 3.21\%$  to  $15.17\% \pm 7.79\%$ ;  $P$ -value = 0.045%).

**Conclusion:** Our results showed that short abstinence may not be beneficial in all infertile males, and it should only be used in selected patients with abnormal DNA integrity.

**Keywords:** CMA3; male infertility; short abstinence; sperm DNA integrity; TUNEL

### INTRODUCTION

Male fertility and sperm production ability have been traditionally assessed using conventional semen analysis<sup>(1)</sup>. The latest World Health Organisation (WHO) guideline<sup>(2)</sup> recommends abstinence of 2 to 7 days prior to semen collection. On the other hand, some reports recommend a single day of abstinence as optimal for semen parameters<sup>(3)</sup>.

In the era of assisted reproductive technology, special consideration needs to be given to sperm DNA integrity along with semen analysis. It has been estimated that 15% of infertile men with normal semen analysis (presumed to have idiopathic infertility) have increased sperm DNA injury<sup>(4)</sup>. A higher level of sperm DNA fragmentation index (DFI) is associated with lower natural pregnancy rate and decreased assisted reproductive technology (ART) outcome<sup>(5)</sup>. In a state of oxidative stress, reducing the epididymal transit effect may lead to better semen quality. This goal may be achieved via two means: short abstinence and testicular sperm retrieval<sup>(6)</sup>.

The effect of short abstinence on sperm DNA have been

widely assessed by different groups, yet controversy remains. Short abstinence is usually considered as abstinence less than 24 hours (instead of recommended abstinence of 2-7 days). Many reports<sup>(6,7)</sup> have shown positive effects for short abstinence on sperm DNA quality and ART outcomes, while others have reported contrasting data<sup>(8,9)</sup>. This discrepancy may be due to selection bias, difference in sample size, or type of the tests used.

In this context, the question to be addressed is whether the standard abstinence period<sup>(2)</sup> is only effective in men with normal sperm DFI or if it is applicable in all infertile males.

The present study assessed the effects of short abstinence (ejaculation after 24 hours of abstinence) on semen and sperm DNA quality in a group of infertile men with previous recurrent pregnancy loss and/or ART failure and elevated sperm DFI.

### MATERIALS AND METHODS

Following the approval by our institutional review board (Ref Number: IR.SSU.RSI.REC.1398.004), 85

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infertile men who provided written informed consent to take part in the study were enrolled (referred to Yazd center for infertility and research, Yazd, Iran, between December 2018 and May 2019). The Yazd center of infertility and research is a high-volume center with approximately 30 outpatient visits per day in the andrology clinic, most of which are referred from all over the country due to recurrent pregnancy loss or ART failure. The primary inclusion criteria were (i) couples with recurrent early abortion (more than two abortions in the first trimester) and (ii) recurrent failed ART (more than three intrauterine inseminations (IUI) or more than two in vitro fertilisation (IVF) sessions and more than two intracytoplasmic sperm injection (ICSI) sessions). Among them, men with disturbed DNA integrity, as assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) test, and/or increased level of chromomycin A3 (CMA3) staining (TUNEL score > 20% and/or CMA3 score > 30) were selected<sup>(10)</sup> and treated with antioxidant medical therapy for 3 months (folic acid 1 mg/day, once daily selenium plus-EuRho® Vital Selen Plus capsule, Euro OTC Pharma GmbH, and once daily /250 mg L-carnitine ). Because these tests have not been standardised yet, the cutoff values were chosen based on previous studies (10-14). At the end of the third month, the participants provided us with two semen samples as described below (in the semen collection section).

Exclusion criteria were (i) a history of smoking, (ii) opium addiction, (iii) multiple sexual partners, (iv) known hormonal abnormality, (v) clinically detected varicoceles, or (vi) cryptorchidism. The same andrologist performed a complete physical examination, evaluated the past medical history and recorded the results. Finally, 21 patients were excluded, and 64 patients were enrolled.

### Semen collection

Each patient provided three semen samples: (i) the first sample before antioxidant medical therapy (with abstinence of 2–7 days based on the WHO recommendation) was considered as untreated sample; (ii) the second sample (treated sample ) after 3 months of antioxidant medical therapy (with abstinence of 3 days) and (iii) the third sample with 24 hrs of abstinence after the second sample, was considered as the short abstinence (short ejaculation) sample.

### Semen analysis and sperm DNA integrity

Semen samples were obtained and analysed based on WHO guidelines (2010)<sup>(2)</sup>. Motility was classified as (i) progressive, (ii) nonprogressive and (iii) immotile. Sperm morphology was reported based on strict criteria. Two different sperm DNA and chromatin tests were performed on each semen sample: (i) a terminal deox-

ynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) test, which directly measures sperm DFI, and (ii) chromomycin A3 staining (CMA3), which evaluates sperm DNA protamination (or compaction).

### TUNEL test

In brief, after fixation with paraformaldehyde, the samples were washed and treated with PBS and a mixture of methanol and 3% H<sub>2</sub>O<sub>2</sub>.

In the next step, after being immersed in Triton X-100 and sodium citrate, the samples were washed with PBS and then stained with a mixture of enzyme and fluorescently labelled dUTP solution. Finally, they were assessed using an In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) and fluorescence microscopy (BX51, Olympus, Tokyo, Japan)<sup>(15)</sup>. At least 200 sperms were counted and considered as containing damaged DNA if they turned bright green.

### Chromomycin A3

CMA3 is a guanine–cytosine binding fluorochrome which competes with protamine for the same binding locus. Therefore, CMA3 staining assesses chromatin integrity (protamination). Briefly, air-dried samples were fixed with Carnoy's solution, stained with CMA3 for 20 min, rinsed and then mounted with glycerol buffer, and finally stored at a low temperature overnight. The next morning, the samples were assessed using a fluorescence microscope<sup>(16)</sup>.

### Sample size calculation

Considering the following formula, the estimated sample size for this before-after study, was 64 samples:

$$n \geq \frac{(z_{\alpha} + z_{\beta})^2 \sigma^2}{(\mu_1 - \mu_2)^2}$$

Where type I error rate ( $\alpha$ ), *effect size*  $(\frac{\mu_1 - \mu_2}{\sigma})$  and power ( $1 - \beta$ ) were 0.05, 0.35 and 0.8, respectively<sup>(17)</sup>

### Statistical methods

Continuous variables are reported as mean (standard deviation) and categorical data are presented as frequency (percentage). In order to assess the difference between two variables for the same subject, paired sample T test was used for continuous variables. Also the association between age, body mass index, smoking, alcohol consumption, duration of infertility and the results of sperm DFI were assessed using repeated measures ANOVA. P-value < 0.05 was considered as statistically significant. Statistical analysis was performed using SPSS version 25.0 (IBM, Chicago, Illinois, USA).

**Table 1.** Effect of antioxidants on semen parameters, sperm DNA integrity and chromatin compaction

| Variables              | Untreated Group (N= 64) Mean ± SD | Treated Group (N=64) Mean ± SD | P-value |
|------------------------|-----------------------------------|--------------------------------|---------|
| Volume (ml)            | 3.40 ± 1.41                       | 3.62 ± 1.44                    | 0.161   |
| Concentration (mil/ml) | 67.51 ± 44.40                     | 56.09 ± 37.85                  | 0.005*  |
| Progressive (%)        | 39.69 ± 12.50                     | 38.76 ± 11.85                  | 0.512   |
| Nonprogressive (%)     | 10.55 ± 4.26                      | 10.73 ± 3.34                   | 0.787   |
| Immotile (%)           | 50.02 ± 10.75                     | 50.47 ± 11.59                  | 0.746   |
| Morphology (%)         | 3.54 ± 1.86                       | 3.50 ± 1.66                    | 0.837   |
| TUNEL (%)              | 24.56 ± 9.49                      | 20.64 ± 10.28                  | 0.013*  |
| CMA3 (%)               | 48.75 ± 15.96                     | 47.79 ± 20.78                  | 0.769   |

\*P-value < 0.05 considered as significant

**Table 2.** Effect of short abstinence on semen parameters and sperm DNA integrity and chromatin compaction after previous medical therapy in all the patients.

| Variables          | Treated Group (N=64) Mean ± SD | Short Abstinence (N=64) Mean ± SD | P-value |
|--------------------|--------------------------------|-----------------------------------|---------|
| Volume (ml)        | 3.62 ± 1.44                    | 2.92 ± 1.43                       | <.001   |
| Count (mil/ml)     | 56.09 ± 37.85                  | 54.02 ± 37.51                     | 0.699   |
| Progressive (%)    | 38.76 ± 11.85                  | 38.42 ± 11.32                     | 0.887   |
| Nonprogressive (%) | 10.73 ± 3.34                   | 11.32 ± 4.10                      | 0.245   |
| Immotile (%)       | 50.47 ± 11.59                  | 50.08 ± 9.58                      | 0.656   |
| Morphology (%)     | 3.50 ± 1.66                    | 3.38 ± 1.43                       | 0.729   |
| TUNEL (%)          | 20.64 ± 10.28                  | 17.38 ± 8.59                      | 0.028*  |
| CMA3 (%)           | 47.79 ± 20.78                  | 41.92 ± 18.49                     | 0.019*  |

\*P-value < 0.05 considered as significant.

## RESULTS

A total of 64 male patients were evaluated with a mean age of  $34.7 \pm 4.66$  yrs (range: 27–49 yrs).

The data indicate that antioxidants have statistically significant effects on mean sperm concentration (untreated,  $67.51 \pm 44.40$  million/ml, vs. treated,  $56.09 \pm 37.85$  million/ml;  $P$ -value = 0.005) and the mean TUNEL score (untreated,  $24.56\% \pm 9.49\%$ , vs. treated,  $20.64\% \pm 10.28\%$ ;  $P$ -value = 0.013). However, there was no significant relationship between antioxidant treatment and other semen parameters, including CMA3 staining (Table 1).

The effects of short abstinence on semen parameters and sperm DNA integrity are demonstrated in Table 2. Short abstinence had a statistically significant negative effect on semen volume (recommended abstinence,  $3.62 \pm 1.44$  ml, vs. short abstinence,  $2.92 \pm 1.43$  ml;  $P$ -value < 0.001) but positive (decreasing) effects on TUNEL score (recommended abstinence,  $20.64\% \pm 10.28\%$ , vs. short abstinence,  $17.38\% \pm 8.59\%$ ;  $P$ -value = 0.028) and CMA3 score (recommended abstinence,  $47.79\% \pm 20.78\%$ , vs. short abstinence,  $41.92\% \pm 18.49\%$ ;  $P$ -value = 0.019). Its effect on other semen parameters was not statistically significant ( $P$ -value > 0.05).

Antioxidant drugs may improve ( decreased TUNEL test) or may have no effect on DNA integrity (TUNEL test remains constant or even increase ). Therefore af-

ter three months of antioxidants medical therapy, the samples may have TUNEL score below (responder) or above (non-responder) normal cutoff ( 20%). In this step, we tried to assess the effect of short abstinence on sperm with normal TUNEL score (< 20%) and sperm with abnormal DFI( TUNEL score > 20%)

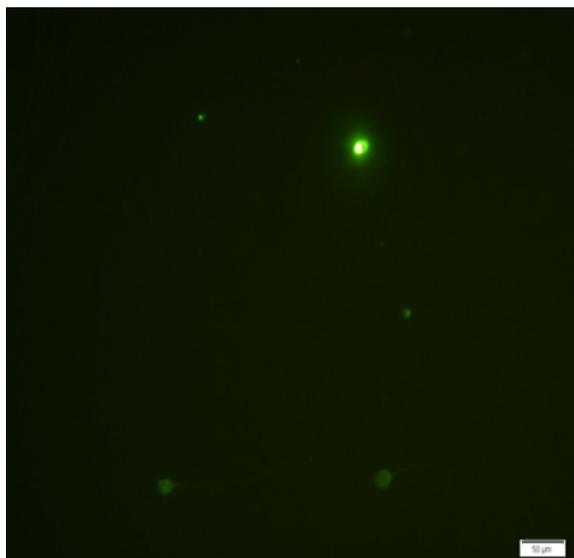
For further robust evaluation of the effects of short abstinence on sperms with normal or increased sperm DNA integrity, table 2 were re-analysed based on a TUNEL cutoff score of 20% as proposed by Sharma and the colleagues.<sup>(10)</sup>, and the results are presented in Table 3.

At the TUNEL cutoff score of 20%, 28 samples had low (TUNEL score < 20%) and 36 others had high (TUNEL score > 20%) sperm DFI. Short abstinence had a negative (increasing) effect on samples with low sperm DFI (mean TUNEL score increased from  $11.89\% \pm 3.21\%$  to  $15.17\% \pm 7.79\%$ ;  $P$ -value = 0.045) and a positive (decreasing) effect on samples with abnormal (high) sperm DFI (mean TUNEL score decreased from  $27.85\% \pm 8.32\%$  to  $19.14\% \pm 8.90\%$ ;  $P$ -value < 0.001).

## DISCUSSION

The present study showed that a short abstinence period could improve sperm DNA integrity in patients with increased sperm DNA damage. To the best of our knowledge, the present study is the first to show that short abstinence may have a paradoxical effect on sperm DFI as evidenced by baseline DNA integrity status. In subjects found to have low DNA damage using TUNEL test (TUNEL score < 20%), short abstinence not only failed to cause further improvement but also had certain deleterious effects. Therefore, short abstinence is probably not applicable to all cases (as recommended elsewhere<sup>(3)</sup>) and should be reserved for selected infertile men with increased DFI.

In a functioning spermatogenesis system, epididymis may act as a screening tool and may eliminate immature sperms due to its oxidative stress effects (induced by epididymis epithelial cells, leukocytes, immature sperms themselves, etc.). Therefore, epididymal passage may lead to subsequent improved semen quality and seminal sperm DFI. In such systems, decreasing epididymal passage time may result in increased sperm DFI as explained in the current study (and in patients with normal sperm DFI). On the other hand, in a mal-functioning system ( e.g. due to incomplete protamination), epididymal passage may lead to additional stress on the vulnerable sperms and therefore may induced sperm DNA injury apart from oxidative stress. In such systems, short abstinence or testicular sperm extraction may have great benefit in preserving sperm DNA in-



**Figure 1.** TUNEL test, sperm with injured DNA depicted as bright green.

**Table 3.** Effect of short abstinence on sperm DNA integrity based on baseline low or high level of sperm function tests (TUNEL test).

| Variables                    | Treated Group (N=64) Mean ± SD | Short Abstinence Mean ± SD | P-value |
|------------------------------|--------------------------------|----------------------------|---------|
| TUNEL < 20% (n = 28 samples) | 11.89 ± 3.21                   | 15.17 ± 7.79               | 0.045   |
| TUNEL > 20% (n = 36 samples) | 27.85 ± 8.32                   | 19.14 ± 8.90               | <.001   |

tegrity.

Nevertheless, in the current study, short abstinence had statistically significant effects on semen volume. It also decreased sperm concentration, percentage of immotile and progressive sperms, sperms with normal morphology and the percentage of non-progressive motility, although with no statistical significance ( $P$ -value > 0.05). In the case of a longer abstinence period, three important events occur. Sperms are stored in the epididymis (with presumable stress effects)<sup>(18)</sup>, the seminal level of reactive oxygen species (ROS) may increase<sup>(8)</sup> and the antioxidant capacity of axillary sexual glands secretions (prostate and seminal vesicle) may decrease significantly<sup>(19)</sup>. These changes may indicate better semen quality in an efficient spermatogenesis process (act as a screening tool) but may lead to increased sperm DNA fragmentation and subsequent infertility in a non-efficient system which is pathologically vulnerable to environmental stressors (e.g. due to abnormal sperm chromatin compaction) or in the oxidative stress status (excessive production of ROS or decreased levels of seminal antioxidants). Multiple ejaculations with short abstinence periods may increase semen antioxidant capacity and decrease seminal ROS, especially those originating from the epididymis<sup>(6)</sup>.

There are a few inconsistent reports regarding the effects of a short abstinence period on semen quality; the discrepancies may be due to differences in the number of recruited participants, fertility status and inclusion/exclusion criteria.

Agrawal and his colleagues, in their study on seven

healthy men with unproven fertility, showed that sperm DFI increased concomitantly with an increase in the abstinence period. They reported that mean sperm DFI increased from 9.9% in cases of short abstinence period (less than 2 days) to more than 17% in cases with long abstinence period (9–11 days). Their conclusion should be cautiously interpreted as their cohort included only a small number of normospermic men<sup>(6)</sup>.

On the other hand, a study conducted by De Jong and colleagues on 11 healthy volunteers and another study by Mayorga-Torres and colleagues on six healthy volunteers, using SCSA techniques, found that a shorter abstinence period might not affect sperm DFI<sup>(9,20)</sup>. Furthermore, De Jong et al. reported that a shorter abstinence period might negatively affect sperm chromatin compaction, making them susceptible to environmental stressors (not observed in the present study). This difference may be due to differences in sample size, type of sperm DFI test used, and fertility status of the recruited men. Even if the small sample size in the above study is not considered, results obtained from healthy participants cannot be attributed to subfertile and infertile men.

Our findings are in agreement with those of Pons and co-workers and Sanchez and colleagues, who used the sperm chromatin dispersion test and reported positive effects of short abstinence on semen quality in 36 and 40 infertile men, respectively<sup>(21,22)</sup>.

The result of previously published studies (in Pubmed) on the effect of a short abstinence period on sperm DFI are summarized in **Table 4**.

**Table 4.** the results of the previous studies about the effects of short abstinence on Sperm DNA.

| Study                           | Number of participants | Abstinence time      | DFI assessment   | Chromatin compaction assessment | Semen volume  | Sperm concentration | Progressive motility | morphology    |
|---------------------------------|------------------------|----------------------|------------------|---------------------------------|---------------|---------------------|----------------------|---------------|
| De jong et al. 2004(9)          | 11                     | 24 hours             | SCSA: No change  | SCSA(HDS): increased            | decreased     | decreased           | No change            | No change     |
| Gosalvez et al, 2011 (29)       | 33                     | 3 hours and 24 hours | SCD: decreased   | Not mentioned                   | Not mentioned | Not mentioned       | Not mentioned        | Not mentioned |
| Pons et al. 2013 (21)           | 34                     | 24 hours             | SCD: decreased   | Not mentioned                   | decreased     | decreased           | decreased            | Not mentioned |
| Sanchez Martin et al. (22)      | 21                     | 12 hours             | SCD: decreased   | Not mentioned                   | decreased     | No change           | No change            | Not mentioned |
| Mayorga Torres et al.(2015)(20) | 6                      | 24 hours             | SCSA: no change  | Not mentioned                   | decreased     | No change           | No change            | Not mentioned |
| Mayorga Torres et al.(2016)(8)  | 3                      | 2 hours              | SCSA: decreased  | Not mentioned                   | decreased     | decreased           | decreased            | Not mentioned |
| Agrawal et al. 2016(6)          | 7                      | 24 hours             | TUNEL: decreased | Not mentioned                   | decreased     | decreased           | No change            | No change     |
| Uppangala et al. 2016 (30)      | 16                     | 24 hours             | SCD: decreased   | Aniline blue: increased         | decreased     | decreased           | No change            | No change     |
| Comar et al, 2017(31)           | 2458                   | Less than 2 days     | TUNEL: decreased | CMA3:increased                  | decreased     | decreased           | increased            | No change     |
| Shen et al., 2018(32)           | 167                    | 1-3 hours            | SCSA: decreased  | SCSA:increased                  | decreased     | increased           | increased            | Not mentioned |
| Borges et al. 2019 (7)          | 818                    | 4.15± 2.72 days      | SCD: decreased   | Not mentioned                   | decreased     | decreased           | No change            | No change     |

The present study also showed that antioxidants, despite showing statistically significant improvement in TUNEL tests, could not remarkably improve chromatin compaction. It may be due to the fact that ROS, although are harmful in high amounts, considered as physiologic in small quantity, and are essential for sperm maturation (chromatin compaction)<sup>(23)</sup>. Antioxidant overuse, especially in cases with normal seminal ROS levels, may eliminate such physiologic amount and may lead to decreased sperm maturation (disturbed chromatin compaction). This is so-called reductive stress phenomenon<sup>(24,25)</sup>. This may be the reason for decreased semen quality after antioxidants therapy in some patients. Measurement of the ROS level has not been performed in our center, possibly leading to antioxidant overprescription and related side effects.

Menezo and co-workers previously reported a similar finding. In their cohort of 54 patients using the SCSA technique, they reported a significant decrease in sperm DFI (32.4% to 26.2%;  $P$ -value < 0.05) and increase in sperm decondensation rate (17.5% to 21.5%;  $P$ -value < 0.05) after antioxidant therapy<sup>(26)</sup>.

Because increased sperm DFI may lead to a lower ART outcome<sup>(27)</sup>, methods that improve sperm DNA integrity appear necessary. Nonresponders to antioxidant therapy (those with disturbed sperm DNA integrity) could be treated with a simple, cost-effective, noninvasive approach (short abstinence) instead of invasive/expensive alternatives (such as testicular sperm extraction, magnetic activated cell sorting and physiologic intracytoplasmic sperm injection).

There are some limitations in the current study. First, the present study showed that short abstinence might improve both sperm DFI and chromatin compaction status, but its probable positive effect on ART outcome remains to be elucidated.

Second, in the current study, the sperm DNA integrity was assessed by TUNEL test and CMA3 staining, using a fluorescence microscope. Since this method may be operator dependent, our results need to be further assessed with flow cytometric techniques, in future studies.

Third, it has been reported that testicular sperms have a higher rate of chromosomal aneuploidy in comparison with that in ejaculated samples<sup>(28)</sup>; the question of whether this is true in cases of short abstinence will be addressed in future studies.

Finally, the present study mainly included men with subfertility (increased DFI with approximately normal semen analysis) and having no control group. Future studies are anticipated assessing the effects of short abstinence on males with abnormal semen parameters and sperm DNA integrity.

## CONCLUSIONS

The present study showed that short abstinence (abstinence time less than 24 hours) would improve sperm DNA integrity in patients with high sperm DFI and previous attempts of failed medical therapy. However, because the effects of such a strategy on ART outcome and chromosomal aneuploidy are unknown, it must be applied cautiously until further investigations verify its safety.

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## DECLARATION OF INTEREST

No potential conflict of interest was reported by the authors.

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