Detection of apoptosis in ejaculated human spermatozoa before and after vapor freezing

Tahmineh Peirouvi PhD¹, Mehrzad Sadaghiani MD¹, Farahnaz Noroozinia MD¹, Siamak Salami PhD¹, Farhang Abed PhD²

¹Department of Histology, Faculty of Medicine, Pardis Nazloo, Urmia University of Medical Sciences, Urmia, Iran. ²Infertility and Reproductive Health Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Abstract The objective of this investigation is to evaluate the effect of vapor freezing on apoptosis of human spermatozoa in infertile and fertile men. Semen samples were collected from infertile (n=25) and fertile (n=10) men after 48h abstinence of intercourse. After standard semen analysis, each semen sample was divided into two aliquots. First aliquot was assessed by TUNEL assay for showing apoptosis before vapor freezing and second aliquot was assessed after thawing. Results were analyzed by Wilcoxon matched pairs test. Mean percentage of apoptotic cells significantly increased in both infertile and fertile men. Apoptotic cells were lower in fertile men than infertile men. DNA fragmentation occurs at head and mid piece of the spermatozoa after freezing and thawing. Therefore freezing and thawing induced fragmentation of DNA spermatozoa and reduced fertility potential of spermatozoa in ART procedures.

Introduction Cryopreservation of spermatozoa is useful for the preservation of reproductive potential in men before chemotherapy, radiotherapy, surgical cancer and in spinal cord injury (Giraud et al., 2000; Donnelly et al., 2001). Cryopreservation induces many alterations in structure and function of spermatozoa, such as decrease in motility, viability and fertilization potential (Duru et al., 2001; Schuffner et al., 2001) due to alterations induced by physical and chemical stress (Paasch et al., 2004). Apoptosis is a form of cell death and is characterized by morphological features such as decrease in cell volume, chromatin condensation, nuclear fragmentation, and DNA fragmentation (Chen et al., 2004). Apoptosis occurs during normal spermatogenesis and play an important role in controlling spermatozoa population (Duru et al., 2001). In addition, apoptosis is induced by various environmental, chemical and physical factors: including X rays, chemotherapeutic drugs and temperature changes (Nagata 1997). TUNEL assay is used for detection of DNA cleavage into double stranded LMW DNA fragments (mono-and oligo nucleosomes) and is sensitive and fast (Shen et al., 2002). In this study we evaluated of vapor freezing effects on apoptosis of spermatozoa in ejaculated semen by using TUNEL assay.

Materials and Methods
Semen samples were obtained from 25 infertile and 10 fertile men. Samples collected by masturbation following 48 hours sexual abstinence. Semen analysis was performed by standard methods (WHO, 1999). Each sample divided into two aliquots for DNA damage analysis by TUNEL assay before vapor freezing and thawing.

Semen were pipetted into cryovials 2 ml (NUNC France) and mixed with spermfreeze™ cryoprotectant (Fertipro NV, Sint-Martens-Latem, Belgium) as per instructions provided. The loaded specimen

Apoptosis in frozen-thawed sperm
was exposed to nitrogen vapor at 20 cm over the liquid nitrogen for 5 minutes, then plunged into liquid nitrogen and transferred to liquid nitrogen storage tank. One month after freezing, specimens removed from liquid nitrogen storage tank, transferred to a water bath at 37°C for 5 minutes. The thawed semen used for detection of DNA damage in the spermatozoa.

Apoptosis was measured by detecting DNA fragmentation using In Situ Cell Death Detection Kit, Fluorocine (Boehringer, Mannheim, Germany). Semen samples containing 20×10⁶ spermatozoa washed 3 times with PBS, the washed spermatozoa is fixed in 2% paraformaldehyde for 30 min at 15-25°C. Cell suspension was centrifuged at 300 g for 10 min and fixative removed. Cell pellet resuspended in permeabilisation solution (0.1% Triton X-100, 0.1% Sodium Citrate; Aldrich) for 2 min at room temperature. After washing twice with PBS, 50 µl of TUNEL reagent was added to each sample. The positive control for DNA fragmentation was detected by the same TUNEL technique. No deoxuryridine-5'-triphosphate was added for the negative control. After incubation at 37°C for 60 min in the dark, apoptotic cells were detected by fluorescence microscope (Micros, Austria). The statistical analysis were performed using SPSS software for windows. Wilcoxon matched pairs test used for comparison of data before and after freezing. Results are expressed as Mean ± SD. The difference was considered statistically significant, when P<0.05.

Results
Semen parameters in infertile and fertile men before vapor freezing are shown in Table 1. Fast moving spermatozoa exhibiting progressive motility is lower in infertile men in comparison to fertile men. Fig 1 shows apoptotic cell with DNA damage were observed TUNEL positive (green color). We found that TUNEL stains head and mid-piece of the spermatozoa.

Biochemical event of apoptosis, resulting in cleavage of nuclear DNA into oligonucleosome – sized fragments caused by activation of endogenous endonucleases (Zhang 2002).

Table1. Characteristics of semen parameters before vapor freezing infertile and fertile men.

<table>
<thead>
<tr>
<th>Semen parameters</th>
<th>Infertile men (n=25) Mean ± SD</th>
<th>Fertile men (n=10) Mean ± SD</th>
<th>Normal values (WHO, 1999)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>2.98±1.48</td>
<td>2.57±0.11 0.136</td>
<td>≥2</td>
</tr>
<tr>
<td>Sperm.Conc</td>
<td>47.44±36.28</td>
<td>26.7±0.33 p=0.007</td>
<td>≥20</td>
</tr>
<tr>
<td>Motility (a+b)</td>
<td>32.4±5.08</td>
<td>72.3±13.7 p=0.000</td>
<td>≥50</td>
</tr>
<tr>
<td>Morphology</td>
<td>34.6±8.69</td>
<td>47.5±5.45 p=0.145</td>
<td>≥15</td>
</tr>
</tbody>
</table>

a= progressive and fast motility, b= progressive motility

Discussion
The final stage of apoptosis is DNA fragmentation (Ramos 2001). DNA fragmentation is considered as the key biochemical event of apoptosis, resulting in cleavage of nuclear DNA into oligonucleosome – sized fragments caused by activation of endogenous endonucleases (Zhang 2002).

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Our result showed that apoptotic cells increased significantly after vapor freezing and thawing infertile and fertile men. Our findings also are in agreement with previous study (Oosterhuis & Vermis 2004; Hammadeh et al., 1999) that freezing of spermatozoa for 24 and 48 hours in both fertile and infertile men increases the number of apoptotic cells. Therefore, improvement of cryopreservation techniques would improve quality of sperm preservation in infertile men.

### Table 2. Apoptotic cells before freezing and after thawing from infertile and subfertile men

<table>
<thead>
<tr>
<th>Apoptotic cells</th>
<th>Pre-freeze (Mean±SD)</th>
<th>Post-thaw (Mean±SD)</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subfertile men</td>
<td>6.72±4.79</td>
<td>10.04±5.04</td>
<td>0.000</td>
</tr>
<tr>
<td>Fertile men</td>
<td>3.68±1.09</td>
<td>7.89±2.78</td>
<td>0.012</td>
</tr>
</tbody>
</table>

### Conclusion
DNA fragmentation occurs at head and mid piece of the spermatozoa after freezing and thawing. Therefore freezing and thawing induced fragmentation of DNA spermatozoa and reduced the fertility potential of spermatozoa.

### References:


