

Research Article

SPERM DNA INTEGRITY BEFORE AND AFTER CRYOPRESERVATION

D. Ramesh Raja, Sasikala Natrajamani, Ramakrishnan

Dr.D.Ramesh Raja, Clinical Embryologist and Andrologist, Department of Reproductive Medicine, Chettinad Super Speciality Hospital, Kelambakkam-603103.

Dr.Sasikala Natrajamani, Chief Embryologist, Crea Conceptions, Bharathiraja Hospital, Chennai.

Dr.Ramakrishnan, Genetician. Chettinad Health city.

ABSTRACT

To study the DNA integrity of spermatozoa of infertile men before and after cryopreservation and to establish the difference between four groups of patients namely normozoospermic, oligozoospermic, asthenozoospermic and teratozoospermic men. Type of study: Prospective study. Study place: Reproductive Medicine Department, Chettinad Health City. Data from 40 men of age range 25-45 years were included in the study. The study population was divided into four groups Group A Normozoospermic (n=10), Group B Oligozoospermic (n=10), Group C Asthenozoospermic (n=10), Group D Teratozoospermic (n=10). After semen analysis, each semen sample was mixed with the cryoprotective media and was frozen with liquid nitrogen (-196 ° C). Acridine Orange test was used for the assessment of chromatin structures. After 2 weeks of cryostorage, semen samples were thawed and the effect of cryopreservation on sperm chromatin integrity was evaluated. DNA damage was significantly greater ($p < 0.001$) following Cryopreservation. Group B, C, D had higher DNA damage than group A samples. The freeze-thawing procedure has a negative impact on the DNA integrity of human spermatozoa. Results indicate that sperm DNA damage was significantly higher in groups Oligo, Astheno and Teratozoospermic patients than Normozoospermic patients.

Key words: Cryopreservation, Human Sperm DNA integrity, Acridine Orange test.

INTRODUCTION

Cryopreservation literally means “maintenance of the viability of excised tissues or organs at extremely low temperatures”. Cryopreservation plays an important role in assisted reproduction, as it enables the reproductive cells collected and/or fertilized in one treatment cycle, to be used for in a future treatment cycle. It is also widely used as a method of storing different cell types and tissues including male gametes and occasionally female gametes. Applications include the preservation of spermatozoa before radiotherapy and/or chemotherapy¹, which are likely to lead to

testicular failure or ejaculatory dysfunction. However, due to the damage induced by freezing, the motility of cryopreserved spermatozoa after thawing is significantly reduced and shows wide intra- and inter-individual variability^{2,3}.

Cryopreservation of cells is always encountered with some adverse effects, especially osmotic either during freezing or during thawing. This is also true of spermatozoa, where damage to the cell membrane and impairment of sperm motility resulting from freezing or thawing process lower the fertilizing capacity. The post thaw motility of human sperm can range from 20 percent to 50 percent of the sperm motility

before cryopreservation⁴. The loss of motility is believed to be caused by several factors, including diminished integrity of the membranes and cryodamage to the membranes of the intracellular organelles, which then affect energy metabolism and synthesis. Studies show that cryopreservation of semen also induces DNA damage which can prove highly detrimental to the resulting embryos^{5,6,7}. Poor chromatin packing has been shown to correlate with numerous adverse reproductive outcomes including Poor fertilization after IVF and ICSI⁸ and increased incidence of pregnancy loss⁹.

Sperm DNA integrity is essential for accurate transmission of genetic information. This parameter has been shown to be of paramount importance for fertilization and normal embryo development^{7,9,10,11}. The origin and impact of sperm DNA fragmentation has thus been a subject of numerous studies. Infertile men with poor sperm motility and morphology have increased DNA fragmentation compared to individuals with normal semen parameters^{12,13,14}. Men with normal semen analysis may also have a high degree of DNA fragmentation, which can be a major cause of unexplained infertility and is not detected during a routine semen analysis. Sperm DNA fragmentation may result from aberrant chromatin packaging during spermatogenesis^{15,16,17}, defective apoptosis before ejaculation¹⁸, or excessive production of reactive oxygen species (ROS) in the ejaculate^{12,19}.

There is mounting evidence that sperm DNA damage is common in infertile men. Also, the increasing concern over genetic and epigenetic abnormalities in children conceived through assisted conception urges us to explore the subject of human sperm genomic integrity further^{20,21}. Exposure to environmental or industrial toxins, oxidative

stress, smoking, etc, are known to cause sperm DNA fragmentation and infertility^{22,23}. Fertilization by sperm containing fragmented DNA may lead to foetal mutations or malformations and may also increase the risk of cancer in offspring²³. Moreover, fertilization by sperm with fragmented DNA results in poor embryonic development, decreased implantation, lower pregnancy rates, and recurrent pregnancy losses^{10,21,23,24}. Therefore, it would seem logical to evaluate the chromatin integrity of sperm in infertile men before assisted reproduction techniques are used. Sperm DNA damage can also be induced by cryopreservation since the gametes are subjected to extreme temperatures and hence extreme changes in their internal milieu. It is highly essential to know the impact of cryopreservation on the DNA integrity of spermatozoa since it is a routine technique used in assisted reproduction.

Different techniques have been used to assess sperm DNA integrity including direct methods such as single cell electrophoresis and Terminal deoxynucleotidyl transferase-mediated DUTP nick end labeling assay (TUNEL). Impaired DNA integrity can be detected using the in situ DNA denaturation test with acridine orange^{13,25} because damaged DNA is more prone to denaturation by heat or low pH than undamaged DNA. These methods require expensive equipment and elaborate procedures which are time consuming and give similar results as other inexpensive staining techniques like acridine orange staining. Hence use of Acridine Orange test may be a more practical alternative to the expensive tests.

The human sperm DNA integrity assessed by Acridine Orange stain^{3,7} has been widely used for evaluation of male infertility.

OBJECTIVE

The objective of this study was to assess the impact of cryopreservation on the DNA Integrity of spermatozoa in infertile men using Acridine Orange.

METHODOLOGY

Type of study: Prospective study

Study place:

Department of Reproductive Medicine, Chettinad Hospital and Research Institute.

Study population:

40 consenting patients (age range: 25 to 45 years) who attended the clinic during May and June 2010 were included in the study. The subjects were divided into four groups (10 in each group):

- Group A Normozoospermic
- Group B Oligozoospermic
- Group C Asthenozoospermic
- Group D Teratozoospermic

Patients with history of vasectomy, increased round cells in semen, h/o smoking, alcohol use, substance abuse, and diabetes were excluded from the study.

STUDY DESIGN**Semen collection**

All men were instructed to collect semen by masturbation into a sterile plastic specimen cup. They were instructed to abstain from ejaculation for 48-72 hours before producing the semen sample. They were also advised to refrain from using any lubricants or drugs for facilitation of ejaculation.

Semen samples were permitted to liquefy for no more than 60 minutes at 37°C before

analysis. A single semen sample was collected from every individual.

Semen analysis

Sperms were counted in a Makler chamber[®] (SEFI Medical Instruments Ltd.). The sperm motility was assessed after liquefaction by grading the sperm cells as

Progressive Motility

Non Progressive Motility

Immotile

Sperm morphology scores were determined by the Tygerberg “strict” criteria (Kruger et al, 1986, 1988). All the other parameters analyses were performed in accordance with the guidelines of the World Health Organization by a single observer.

NORMAL REFERENCE VALUES (WHO 2010 – Laboratory manual for the Examination and processing of human semen- fifth edition)

Concentration 15 million/ml.

Total sperm count 39 million/ ejaculate.

Motility $\geq 32\%$ or more with a progressive motility or $\geq 40\%$ of total motility (Progressive and Non progressive motility) within 60 minutes of ejaculation.

Morphology $\geq 4\%$ normal forms.

Cryopreservation of spermatozoa

Semen was mixed with Spermfreeze (Fertipro 8730 Beernem, Belgium) cryoprotectant at a ratio of 1:1 in a sterile container. The cryoprotectant (stored at 4°C) was allowed to equilibrate to room temperature before use and was added gradually with gentle swirling. The mixture

was then left at room temperature for 10 min and then filled in cryovials and frozen by static phase vapour cooling. Aliquots were suspended in liquid nitrogen vapour 10cm above the level of liquid nitrogen (-80°C) for 15min. The samples were then plunged into liquid nitrogen (-196°C) and stored for at least two weeks.

Thawing of spermatozoa

The cryovials containing spermatozoa were removed from liquid nitrogen and the caps were loosened to prevent them from exploding. The samples were then left to thaw at room temperature for 15–20 min. Once the samples thawed, the contents were transferred to a sterile container. An equal volume of sperm wash media was added gradually to avoid osmotic shock. The fluid was then centrifuged at 2000rpm for 10 min. The supernatant was removed and the pellet was resuspended in an appropriate volume of sperm wash media.

Sperm chromatin integrity by Acridine Orange Fluorescence

A smear was made from each semen sample and placed on a clean glass slide on the day of the analysis. This was fixed overnight in freshly prepared Carnoy's solution (3 parts of Methanol and 1 part of Glacial acetic acid).

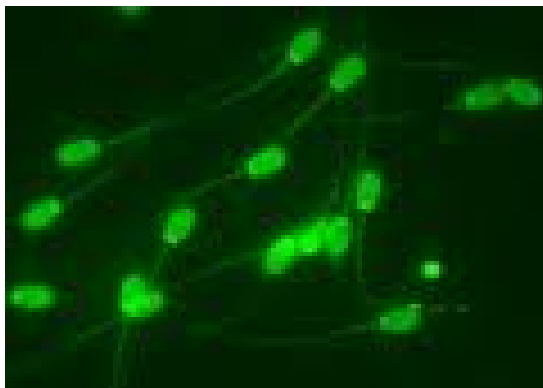
The next day slides were air-dried and stained with AO solution (10 ml of 1% AO in distilled water added to a mixture of 40 ml of 0.1M Citric acid and 2.5 ml of 0.3M Na₂HPO₄, 2H₂O) for 5 min.

The slides were then gently rinsed in distilled water and kept in a cool and dark place until evaluation was carried out.

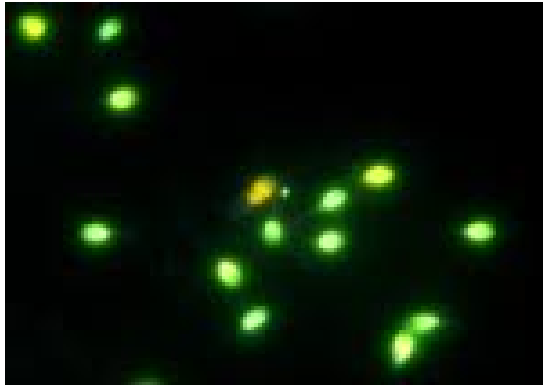
At least 400 spermatozoa were counted per slide under a fluorescent microscope (BX41; Olympus, Tokyo, Japan) under x400 magnification, with excitation at 450–490 nm. Proportion of sperms with fragmented DNA was calculated for each sample before and after cryopreservation.

Spermatozoa with normal, intact double-stranded DNA stain green and those with denatured ones show red or orange fluorescence (Pictures 1-3).

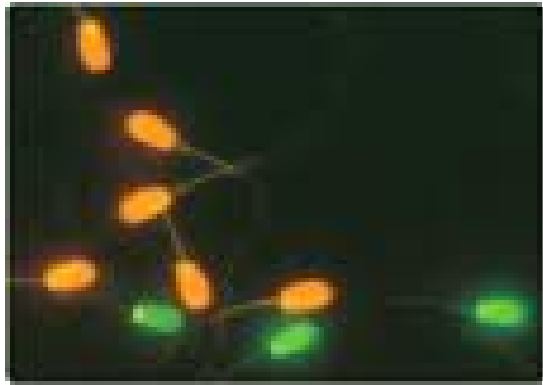
Picture 1: AO stained slide before cryopreservation



Picture 2: AO stained slide after cryopreservation



Picture 3: AO stained slide showing high proportion of DNA fragmentation.



STATISTICAL ANALYSIS

All analyses were conducted using the statistical package SPSS 10.1. The one-way ANOVA test was used for comparison of mean values within the four groups studied. Paired sample test was used to determine the differences in before and after cryopreservation of four groups.

RESULTS

The mean of percentage of sperms with DNA fragmentation before cryopreservation was calculated for each group. The values are as follows:

- Group A: 8.7%
- Group B: 9.8%
- Group C: 10.2%
- Group D: 9.5%

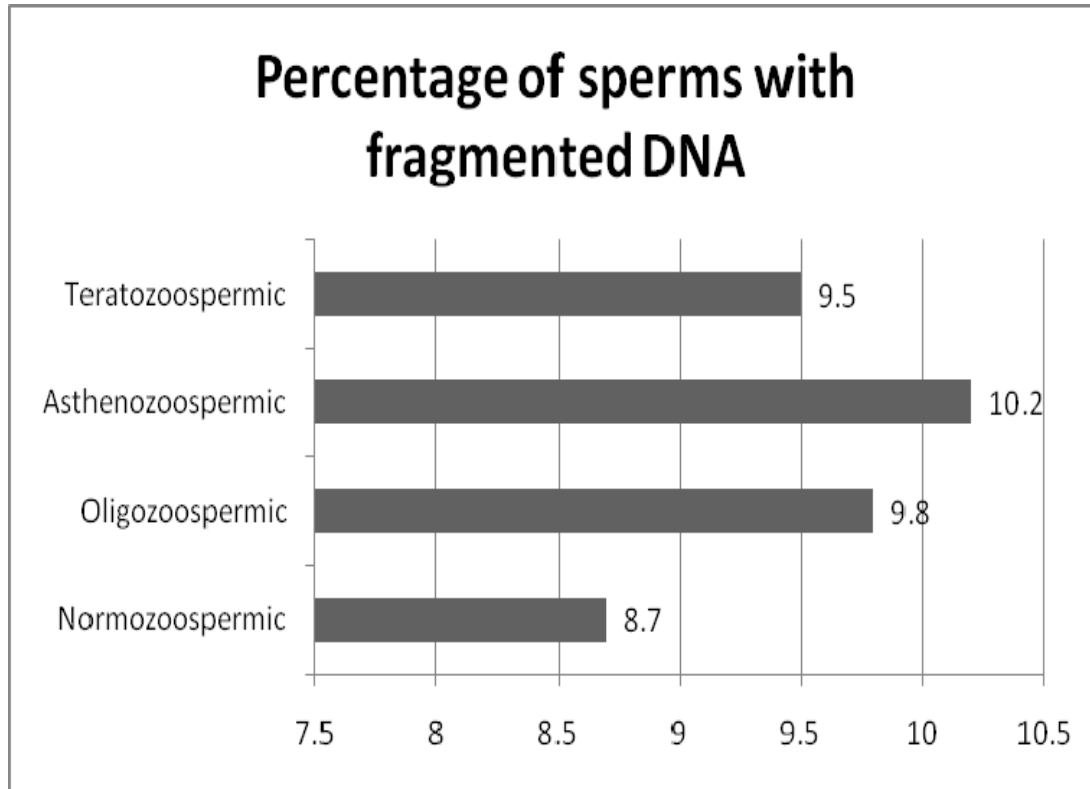


Fig: 1 – Showing the mean DNA damage of four groups before cryopreservation

The highest percentage was in the asthenozoospermia group and the lowest was in the normozoospermic men.

Percentage of DNA damage in each of the four groups was repeated after Cryopreservation. The values are as follows:

Group A: 13.9%

Group B: 18.9%

Group C: 21.6%

Group D: 22.7%

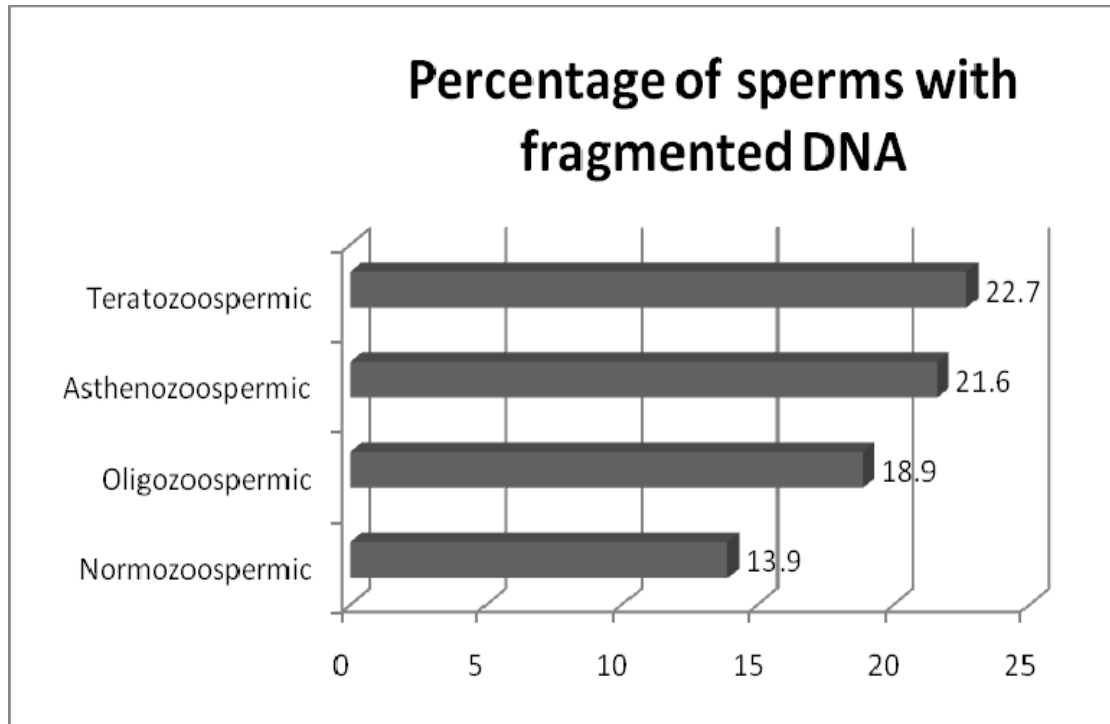


Fig: 2 – Showing the mean DNA damage of four groups after cryopreservation

Again the lowest levels are in the men with normozoospermia but the highest is in the teratozoospermic men. Fragmented DNA in the asthenozoospermic men was only slightly lower at 21.6%.

The difference in the mean percentage of sperms with DNA damage was then calculated:

Group A: 5.2%

Group B: 9.1%

Group C: 11.4%

Group D: 13.2%

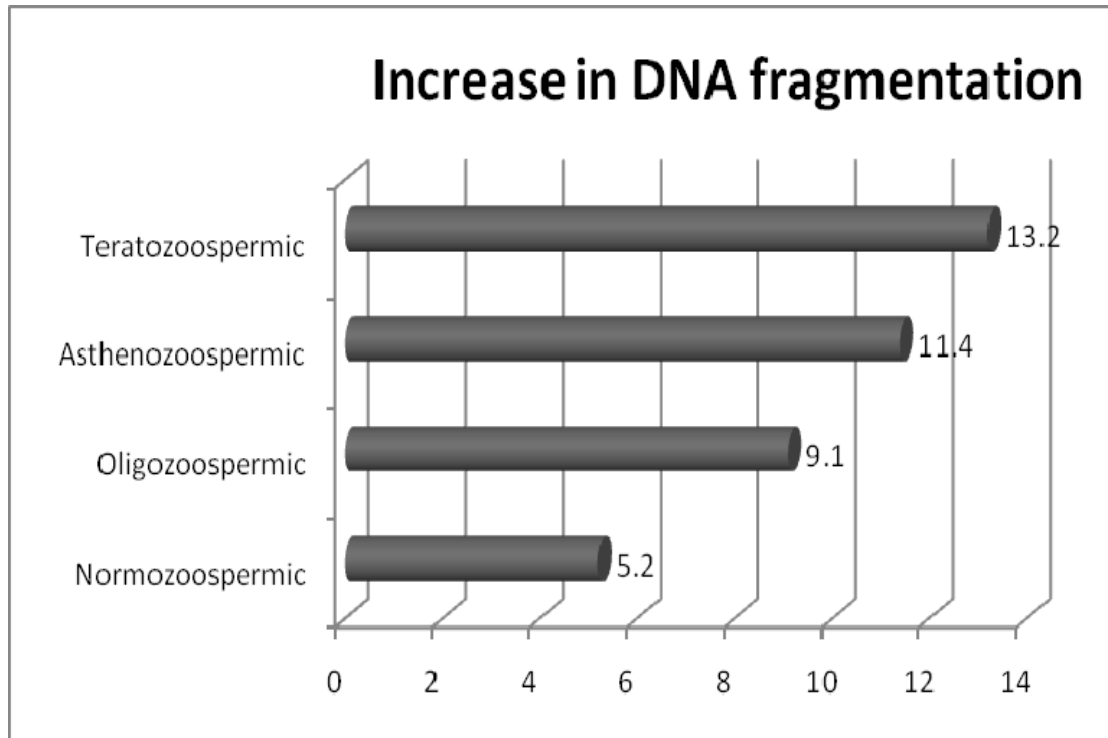


Fig: 3 – Showing the mean DNA damage of four groups due to cryopreservation

The increase in the DNA fragmentation due to cryopreservation was the most in the teratozoospermic group and was least in the normozoospermic group.

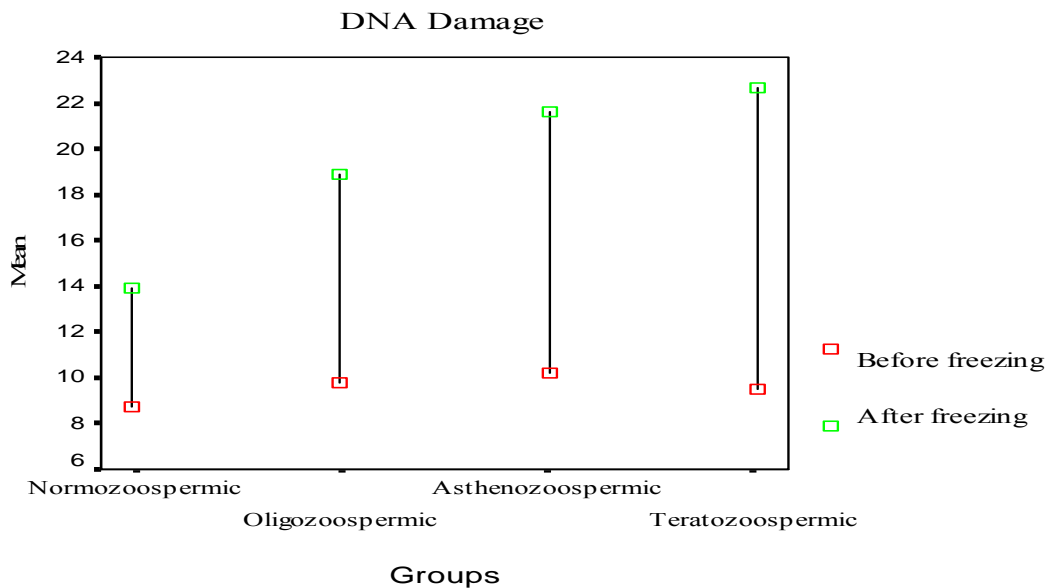


Fig: 4 - Showing DNA damage before and after freezing (cryopreservation):

One way ANOVA test was used to compare the mean value of four groups. DNA damage after cryopreservation within each group was statistically ($p=0.000$) significant.

Table: 1 - One way ANOVA – Comparison of Mean values of the FOUR groups (Shaded area showing statistically significant results)

		Sum of Squares	df	Mean Square	F	Sig.
DNA Damage before freezing	Between Groups	12.100	3	4.033	.361	.781
	Within Groups	401.800	36	11.161		
	Total	413.900	39			
DNA Damage after freezing	Between Groups	461.675	3	153.892	11.984	.000
	Within Groups	462.300	36	12.842		
	Total	923.975	39			

Paired sample test was used to determine the differences in DNA fragmentation between the four groups before and after cryopreservation.

Table: 2- Paired Samples Statistics

Group		Mean	N	Std. Deviation	Std. Error Mean
Normozoospermic	DNA Damage before freezing	8.70	10	4.473	1.415
	DNA Damage after freezing	13.90	10	4.433	1.402
Oligozoospermic	DNA Damage before freezing	9.80	10	3.011	.952
	DNA Damage after freezing	18.90	10	2.132	.674
Asthenozoospermic	DNA Damage before freezing	10.20	10	2.530	.800
	DNA Damage after freezing	21.60	10	2.989	.945
Teratozoospermic	DNA Damage before freezing	9.50	10	3.028	.957
	DNA Damage after freezing	22.70	10	4.270	1.350

The following table shows the statistical analysis of the differences in DNA fragmentation before and after freezing. These were statistically significant in each of the four groups.

Table 3: Paired sample test.

Group	Paired Differences					t	df	Sig. (2-tailed)
	Mean	SD	SE	95% Confidence Interval of the Difference				
				Lower	Upper			
Normozoospermic	-5.20	1.814	.573	-6.50	-3.90	-9.067	9	.000
Oligozoospermic	-9.10	2.079	.657	-10.59	-7.61	-13.842	9	.000
Asthenozoospermic	-11.40	2.875	.909	-13.46	-9.34	-12.538	9	.000
Teratozoospermic	-13.20	2.936	.929	-15.30	-11.10	-14.216	9	.000

DISCUSSION

Semen quality is usually measured by assessing concentration, motility and morphology of spermatozoa (WHO, 1999). These parameters, however, are not able to assess alterations in sperm chromatin organization, such as irregular condensation or DNA damage^{26,27}. For this reason, sperm DNA fragmentation may be considered as a valuable tool during the assessment of semen quality. Various theories have been put forward to explain sperm DNA damage. Some authors think that this damage is due

to incomplete maturation of the gametes caused by flawed topoisomerase II activity⁸; some suggest that the alteration in genetic material is the result of an incomplete apoptotic process^{12, 23,25}, and other authors believe that sperm DNA damage may be the result of excess ROS production²³.

Regardless of sperm DNA damage etiology, the discovery of cases of male infertility stemming from sperm DNA alteration has raised a new issue. In particular, there is

very little information on the possible consequences of fertilization, using sperm with anomalous chromatin organization on embryo development, implantation, gestation and offspring²⁸.

Therefore, simple circumvention of fertilization using ICSI may not overcome all the possible deleterious effects arising from defective sperm DNA.

Cryopreservation causes extensive damage to sperm membranes and decreases the percentage of motile spermatozoa and the velocity of their movement^{29,30,31}. Membrane disruption may be a consequence of liquid phase transition changes and increased lipid per oxidation.

Freeze–thawing of spermatozoa also results in a reduction of sperm metabolism which reduces the number of functional spermatozoa available for assisted conception techniques^{32,33}. Sperm DNA integrity is an important factor in the paternal contribution of human sperm to the fertilization process and the maintenance of pregnancy²³.

This study indicates that sperm DNA damage in infertile males is increased after cryopreservation and sperms with abnormal morphology and low levels of motility have higher DNA damage than motile and normal sperms. In this study Normozoospermic group had a lower percentage of sperm with DNA damage compared to the other groups. Several studies have reported similar findings but have suggested different techniques used to find DNA damage.

Although the extent of DNA damage is closely related to sperm function and male infertility, the origin of such damage is still controversial. It is believed that despite improper packaging and ligation during sperm maturation and germ cell apoptosis, oxidative stress is an important factor in

sperm DNA damage²⁴. This is important as mature spermatozoa with DNA damage may exhibit lower functional potential and this may explain the patients' subfertility status especially in couples with so called unexplained infertility³⁴.

This close relationship is particularly important in ICSI, where the sperm introduced is often selected by an operator, the sperm parameters are often 'critical' and, consequently, the risk of injecting sperm with damaged DNA into the oocyte is increased.

CONCLUSION

Cryopreservation induces significant DNA damage in semen samples. Acridine Orange test is the simple economical test and may be useful in predicting DNA fragmentation when compared to other TUNNEL assay and COMET assay and can be used as a routine test prior to ART procedures. The development and correct usage of methods for selecting sperm with undamaged DNA should be studied carefully, especially where ICSI is strongly recommended.

REFERENCES

1. Sheikh N, Amiri I, Farimani M, et al. Correlation between sperm parameters and sperm DNA fragmentation in fertile and infertile men. *Iranian Journal of Reproductive Medicine* 2008;6(1):13-18.
2. Yildiz C, Ottaviani P, Law N, et al. Effects of cryopreservation on sperm quality, nuclear DNA integrity, in vitro fertilization, and in vitro embryo development. *Reprod* 2007; 133:585–595.
3. Chohan KR, Griffin JT, Marine. Comparison of Chromatin Assays for DNA Fragmentation Evaluation in Human Sperm. *J Androl* 2006;27(1):53-59.
4. Shibahara H, Onagawa T, Ayustawati, Jorsaraei S, Hirano Y, Suzuki T, Takamizawa S, Suzuki M. Clinical significance of the Acridine Orange test performed as a routine examination: comparison with the CASA estimates and strict criteria. *Int J Androl.* 2003 Aug; 26(4):236-41.

5. Claassens OE, Menkveld R, Franken DR, Pretorius E, Swart Y, Lombard CJ, and Kruger TF. The Acridine Orange test: Determining the relationship between sperm morphology and fertilization in vitro. *Hum Reprod* 1992;7:242–247.
6. Hoshi K, Katayose H, Yaganida K, Kimura Y, Sato A. The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. *Fertil Steril* 1996 ;66:634–639.
7. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP. Utility of the sperm chromatin structure assay (SCSA) as a diagnostic and prognostic tool in human fertility clinic. *Hum Reprod.* 1999; 14:1039–1049.
8. Bianchi PG, Manicardi GC, Urner F, Campana A and Sakkas D. Chromatin packaging and morphology in ejaculated human spermatozoa: evidence of hidden anomalies in normal spermatozoa. *Mol Hum Reprod* 1996; 2,139–144.
9. Boe-Hansen GB, Ersbøll AK, Christensen P. Variability and Laboratory Factors Affecting the Sperm Chromatin Structure Assay in Human Semen. *J Androl* 2005; 26(3):360-368.
10. Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H and Guérin JF. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod* 2003; 18(5):1023–1028.
11. Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol Hum Reprod* 1996; 2:613-619.
12. Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA, Aitken RJ. DNA integrity in human spermatozoa relationships with semen quality. *J Androl* 2000; 21: 33–44.
- 13 . Agarwal A, Allamaneni SSR. The effect of Sperm DNA damage on assisted reproduction outcomes, a review. *Minireva Ginocol* 2004; 56: 235-245.
14. Sharma RK, Said T, Agarwal A. Sperm DNA damage and its clinical relevance in assessing reproductive outcome. *Asian J Androl* 2004; 6(2):139-148.
15. Gorczyca W, Gong J and Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* 1993;53:1945–1951.
16. Seli E and Sakkas D. Spermatozoal nuclear determinants of reproductive outcome: implications for ART. *Hum Reprod Update* 2005; 11(4):337–349.
17. Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U and Sakkas D. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biol Reprod* 1995; 52,864–867.
18. Sakkas D, Urner F, Bizzaro D, Manicardi GC, Bianchi PG, Shoukir Y and Campana A. Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. *Hum Reprod* 1998;13(4):11–19.
19. Furuki T, Masuko K, Nishimune Y, Obinata M and Matsui Y. Inhibition of testicular germ cells apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. *Development* 1996; 122:1703–1709.
20. Varghese C, Bragais FM, Mukhopadhyay D, et al. Human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics. *Androl* 2009; 41:207-215.
21. Ngamwuttivong T, Kunathikom S. Evaluation of Cryoinjury of Sperm Chromatin According to Liquid Nitrogen Vapour Method. *J Med Assoc Thai* 2007; 90 (2): 224-8.
22. Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 2003; 79 Suppl 3:1597-1605.
23. Aitken RJ, de Luliis GN, McLachlan RI. Biological and clinical significance of DNA damage in the male germ line. *Int J Androl.* 2009; 32:46-56.
24. Sakkas D, Manicardi GC, Bizzaro D and Bianchi PG. Possible consequences of performing intracytoplasmic spermatozoon injection (ICSI) with sperm possessing nuclear DNA damage. *Hum Fertil* 2000a; 3,26–30.
25. Thomson LK, Fleming SD, Aitken RJ, et al. Cryopreservation-induced human sperm DNA

damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod* 2009 Sep; 24(9):2061-70.

26. Sinha Hikim AP, Rajavashisth TB, Sinha Hikim I, Lue Y, Bonavera JJ, Leung A, Wang C and Swerdloff RS. Significance of apoptosis in the temporal and stage-specific loss of germ cells in the adult rat after gonadotropin deprivation. *Biol Reprod* 1997; 57:1193–1201.

27. Richburg JH. The relevance of spontaneous- and chemically-induced alterations in testicular germ cell apoptosis to toxicology. *Toxicol Lett* 2000; 112:11379–11386.

28. Katayose H, Yanagida K, Hashimoto S, Yamada H, Sato A. Use of diamide–acridine orange fluorescence staining to detect aberrant protamination of human-ejaculated sperm nuclei. *Fertil Steril* 2003 Mar; 79 Suppl 1:670-676.

29. Ashkezari MD, et al. The correlation between sperm DNA integrity and IVF success rate in infertile couples. *J Reprod Med* 2006;6(5).

30. Takayama T, Katayose H, Yanagida K, Sato A. Embryo development after intracytoplasmic sperm injection can be predicted by assessment of sperm nuclear chromatin. *Reprod Med Biol* 2009; 8:63-69.

31. Duran EH, Morshedi M, Taylor S and Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. *Hum Reprod* 2002; 17(12):3122–3128.

32. Rodriguez I, Ody C, Araki K, Garcia I and Vassalli P. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J* 1997; 16:2262–270.

33. Petyim S, Choavaratana R. Cryodamage on Sperm Chromatin According to Different Freezing Methods, Assessed by AO Test. *J Med Assoc Thai* 2006; 89 (3): 306-13.

34. Aitken RJ, Baker MA and Sawyer D. Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online* 2003; 7, 65–70.