Australian Journal of Basic and Applied Sciences, 4(5): 835-843, 2010
ISSN 1991-8178

ICSI Outcome after Assessment of Sperm DNA Integrity for Diagnosis of Fertility Potential

[1]Ahmad Kadri, [2]Sayed Bakry, [1]Aziza Mansour, [1]Taimour Khalifa and [2]Usama Sharaf El-Dean

[1]International Islamic Centre for Population Studies and Research (IICPSR)-ART unit, Al-Azhar University.
[2]Zoology Department, Faculty of Science, Al Azhar University, Cairo, Egypt.

Abstract: Rationale: Conventional semen analysis parameters are not sufficient to evaluate male infertility and fertility potential. Now markers of sperm DNA integrity may be better measures of male fertility potential than conventional measures. Sperm DNA damage tests are now available, which has been driven largely by the growing use of assisted reproductive technologies and awareness that the integrity of the male genome plays an important role in IVF. Objective: Our study aimed to evaluate sperm DNA integrity for diagnosis of fertility potential in Egyptian infertile patients with oligo-, astheno- and teratozoospermia, prior to Intra cytoplasmic sperm injection (ICSI), aiming to predict the fertilizing potential, using histochemical technique. Subject and Methods: A total number of 40 men were included in this study, 10 normal fertile men and 30 infertile men with no recognized cause of female infertility. Patients were classified according to the data obtained from routine semen analysis into oligo-, astheno- and teratozoospermia patients. Quantitative measurements of sperm carried out using the image analyzing system. Semen smears were prepared for every patient, the first smear stained with Haematoxylin and Eosin (H/E) for morphological structure, while the second smear stained with methyl green pyronin for localization of nucleic acid (DNA). The obtained histochemical reaction images were calibrated to obtain the optical densities of such reactions by the image analyzing system. Statistical analysis: Student t-test was adopted to compare the obtained optical densities by image analyzing system from normal, oligo-, astheno- and teratozoospermia groups. Results: Head length showed extreme values in cases of teratozoospermia, also tail length was dramatically longer in control group and shorter in all infertile groups. Methyl green pyronin revealed significant decrease of DNA values in all infertile groups, and it is a good indicator to fertility. However, there are some exceptions as some infertile cases may show either normal or higher values. Conclusion: Our data revealed that the assessment of sperm DNA integrity for diagnosis of fertility potential is very important step during the era of ART, especially when using histochemical technique. Also, the dependence on only semen analysis with its routine parameters is not sufficient for infertility evaluation.

Key words: Infertility evaluation, Sperm, DNA, Haematoxlin, Eosin, Methyl Green Pyronin.

INTRODUCTION

Infertility is a tragic condition and defined as the failure to achieve a pregnancy after one year of regular unprotected intercourse with the same partner. The agony and trauma of subfertility is best felt and described by infertile couples themselves. Male infertility is diagnosed when, after testing of both partners, reproductive problems have been identified in the male partner. Male causes for infertility are found in 50% of involuntarily childless couples (WHO, 1999).

Semen analysis is the cornerstone of the evaluation of infertile men for long time by evaluation of semen volume, pH, sperm concentration, morphology and motility for prediction of seminal vesicle and prostate function as well as testicular and genital tract function. Although the routine semen analysis could distinguish between fertile and infertile men, there is significant factors overlap between these groups (Evenson *et al.*, 1999). Moreover, these factors are generally modest predictors of reproductive outcomes. New markers are needed that might be better for accurate diagnosis of infertile from fertile men and that may predict pregnancy outcome and the risk of adverse reproductive events. Conventional semen parameters fall short. There is now

Corresponding Author: Sayed Bakry, Zoology Department, Faculty of Science, Al Azhar University, Cairo, Egypt.
E-mail: sayed.bakry@yahoo.com

some evidence to suggest that markers of sperm DNA integrity may be better measures of male fertility potential than conventional measures, but larger studies are needed to define the clinical value of testing sperm DNA integrity (Spano *et al.*, 2000; Zini *et al.*, 2001and Guzick *et al.*, 2001).The study of sperm DNA damage is particularly relevant in an era where advanced forms of assisted reproductive technologies are frequently used.

The causes of sperm DNA damage, much like those of male infertility and this damage is clearly associated with abnormal spermatogenesis and of course leads to male infertility (Kodama *et al.*, 1997). Several causes of sperm DNA damage were reported by several authors and the most inducer of DNA damage is radio- and chemotherapy. Thus the young men with cancer typically have poor semen quality and sperm DNA damage, even before cancer therapy and are dependent on both duration and dose of exposure which often renders them completely sterile. Since the rapidly dividing germinal epithelium of the testis is a natural target for cytotoxic medications (Sailer *et al.*, 1997 and Banks *et al.*, 2005). Cigarette smoking, direct testicular hyperthermia, varicoceles and hormonal deficiency are associated with a decrease in sperm counts and motility and an increase in abnormal sperm forms and sperm DNA damage (Potts *et al.*, 1999; Zini *et al.*, 2000; Saleh *et al.*, 2003 and Fischer *et al.*, 2003).

Andrologists were exerted huge efforts for several years to understanding the mechanism of sperm DNA damage as the studies carried out by (Ward and Coffey, 1991; Brewer *et al.*, 1999 and Steger *et al.*, 2000). Basically, a mature human sperm has a flat, almond-shaped head contains the nucleus (DNA and proteins), with a cap acrosome (a vesicle rich in hydrolytic enzymes involved in egg penetration). The sperm midpiece represents the proximal part of the sperm tail and is rich in mitochondria. A flagellum propels the sperm, which may live in a woman's reproductive tract for two to three days after sexual intercourse, to the egg. Within the sperm nucleus, there is a histone-rich region that is localized peripherally and a protamine-rich region localized centrally (Zini and Libman, 2006). Sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins (known as histons and protamines) (Ward and Coffey, 1991; Brewer *et al.*, 1999 and Steger *et al.*, 2000).

Protamines are responsible for the compaction and stabilization of the sperm nucleus. It is thought that this nuclear compaction is important to protect the sperm genome from external stresses such as oxidation or temperature elevation. However, high levels of reactive oxygen have been detected in the semen of 25% of infertile men and it has been associated with sperm DNA damage (Irvine *et al.*, 2000).

The condensed and insoluble nature of sperm chromatin protects the sperm during transport of the paternal genome through the male and female reproductive tracts. So, impair of sperm chromatin may causes sperm to loss its genetic integrity or/and induced germ cell mutations which could be transferred to the offspring. (Kosower *et al.*, 1992 and Zini and Libman, 2006). However, the relatively homogeneous nucleoprotein packing of sperm chromatin seen in the light or electronic microscope belies the importance of the unique and complex structure in the proper expression of the paternal genome in early embryo and fetal development (Ward and Zalensky, 1996 and De Jonge, 2000).

The current understanding is that sperm chromatin is tightly packaged by protamines, but up to 15% of the DNA remains packaged by histones at specific DNA sequences. Sperm DNA sequences of histone-bound are less tightly compacted, and it is thought that these DNA sequences or genes may be involved in fertilization and early embryo development (Gatewood *et al.*, 1987 and Gineitis *et al.*, 2000). In comparative study infertile men compared with fertile controls regarding to sperm histone: protamine ratio. The results showed that infertile men have an increased sperm histone: protamine ratio. This increase in nuclear histones reached (~15%) and results in poorer chromatin compaction and a subsequent increased susceptibility to external stresses as temperature elevation in the female reproductive tract and stress (Kosower *et al.*, 1992; Steger *et al.*, 2000 and Oliva, 2006). Also, the association between sperm DNA damage and protamine deficiency suggests that the damage may be due to a defect in spermatogenesis (Evenson *et al.*, 2000). Sperm DNA damage can be measured directly by assessment of fragmentation by using single-cell gel electrophoresis assay or "Comet" assay and by assessment of oxidation using Terminal deoxynucleotidyl Transferase dUTP nick end labeling "TUNEL" assay and liquid chromatography to measure DNA oxidation levels (Kodama *et al.*, 1997 and Aravindan *et al.*, 1997). DNA damage can also be assessed indirectly by means of sperm chromatin integrity assays and by evaluation of nuclear protein levels using nuclear protein stains (e.g., aniline or toludine blue) for detection of histones and DNA stains for detection of denatured or single-stranded DNA (e.g., acridine orange) (Bianchi *et al.*, 1993; Erenpreiss *et al.*, 2001 Spano *et al.*, 2000; Zini *et al.*, 2001 and Guzick, *et al.*, 2001). Both direct and indirect assays are too much expensive for the majority of the infertile patients. So, this study aimed to evaluate sperm DNA integrity for diagnosis of fertility potential in Egyptian infertile patients with oligo-, astheno- and teratozoospermia, prior to Intra cytoplasmic sperm injection (ICSI), aiming to predict the fertilizing capability using more cheaper histochemical technique.

MATERIAL AND METHODS

Patients:

A total of 40 semen samples were collected from couples undergoing ICSI at the Assisted Reproductive unit at the International Islamic Centre for Population Studies and Research (IICPSR)-ART unit, Al-Azhar University, Cairo, Egypt, between October 2007 and October 2008. The informed written consent for use of the semen in this research was obtained from the couples in accordance with the International Islamic Centre for Population Studies and Research (IICPSR)-ART unit, Al-Azhar University, Cairo, Egypt, ethics regulation in research involving the human subjects.

Semen Collection and Preparation:

Briefly, on the day of oocyte aspiration, semen sample was produced on-site by masturbation following sexual abstinence for three to five days in a sterile and wide mouthed container to minimize collection error (Mc Lachlan *et al.,* 2003) and allowed to liquefy at 37*°C* for 20 *min* prior to analysis (Larson, 2001 and Mallidis, 2003). Each sample was divided in two aliquots to be used for analysis of sperm parameters and another part was used for sperm processing to inseminate oocytes via ICSI. The sperm parameters of count, motility and morphology were analyzed according to WHO guidelines using raw semen sample (WHO, 1999; Jeyendran, 2000 and Mortimer *et al.*, 2001). Patients were classified according to the data obtained from routine semen analysis into oligo- , astheno- and teratozoospermia patients.

Histochemical Assay:

Semen smears were prepared for every patient, the first smear stained with Haematoxlin and Eosin (H/E) for morphological structure (Jarow *et al.*, 1985), while the second smear stained with methyl green pyronin for localization of nucleic acid (DNA) (Bancroft and Gamble, 2002).

Quantitative Studies:

Image analyzer system was used for the conduction of the quantitative measurements. A special software program (Quantimet 500 +) was installed in the image system (Leica z model) and through such software program two types of quantitative studies were done.

Semen Processing:

The outcome of intracytoplasmatic sperm injection (ICSI) cycles using fresh sperm samples from oligo-, astheno- and teratozoospermia patients, then washed in 2ml Ham-F10 medium and centrifuged at 1000 rpm for 10 minutes, seminal plasma removed and the sperm in the resulting pellet were allowed to swim up in 1 ml Ham- F10 overlay for 30 mints, enriching the number of motile and morphologically normal sperm cells needed for assisted reproduction. Sperm selection was carried out by the embryologist depends on finding “the best-looking sperm” by eye detected under the inverted microscope (Bartoov *et al.*, 2001).

ART Procedure:

The Controlled Ovarian Hyperstimulation (COH) was performed according to the long luteal suppression protocol, using a GnRHa (Decapeptyl 3.75, Ferring, Germany) and combination of HMG (Menogon, Ferring, Germany) or recombinant FSH (Gonal-F, Serono, Switzerland). The ovulation was induced when at least three follicles had a diameter of $\geq$18 *mm*, using 10,000 *IU* HCG (Choriomon, IBSA, Switzerland). Transvaginal oocyte retrieval was performed by ultrasound guidance. The oocytes containing cumulus cells were collected from clear follicular fluid. Granulosa cells were separated from collected oocytes using enzymatic (Collagenase, Sigma) and mechanical digestion. The ICSI was performed with microinjection equipment (Narishege, Japan) and an inverted microscope (olympus, Japan). Metaphase II oocytes were injected with preferably a motile spermatozoon into the ooplasm. These procedures have been described previously by (Van Steirteghem *et al.*, 1993; Joris *et al.*, 1998 and Al-Hasani *et al.*, 1999).

Assessment of Fertilization, Embryo Cleavage and Establishment of Pregnancy:

The oocytes were assessed for fertilization at 16-18 *hrs* after microinjection (two pronuclear stage, 2PN). The embryos were graded according to their morphology, 48 hrs after insemination. The classification was as follows: grade A: no fragmentation and four regular cells; grade B: <20% fragmentation; grade C: between 21 and 50% fragmentation and grade D: >50% fragmentation. The transfer of the embryos took place either

at 48 or 72 hrs after ICSI, according to the treatment plan of the center. A chemical pregnancy test is done fifteen days after embryo transfer. A clinical pregnancy was determined when at least one foetus with a positive heartbeat was revealed by ultrasonography 6-7 weeks after the transfer (Ebner *et al.*, 2001).

Statistical Analysis:

Mean and SD were calculated and $p < 0.05$ was considered as significant. All statistical analyses were performed using SPSS ver12. Student t-test was adopted to compare the obtained data from normal, oligo-, astheno- and teratozoospermia groups.

RESULTS AND DISCUSSION

In this study, 40 semen samples were collected, the mean age of the male participants ranged from 22-51years and mean age of female participants ranged between 24-38 years. The results of semen analysis, smoking and infertility periods are summarized in Table (1). According to results of semen analysis, there were 10 normal volunteers, 10 Oligospermic patients, 10 Asthenospermic patients and 10 Teratospermia patients were analyzed regarding the routine semen parameters and sperm DNA integrity.

Table 1: Summary of ages, period of infertility some semen parameters in both fertile and infertile groups.

Parameters		Control	Oligospermia	Asthenospermia	Teratospermia
Age	Males	(22-48) 30 years	28-40(36.10 years)	(28-49) 32.7 years	(27-51) 34.6 years
	Females	(24-32) years	(25-37) years	(26-38) years	(28-37) years
Smoking		60% smoker	40% smoker	50 smoker	70% smoker
Sperm Count		(25-126x10^6	5-15x10^6	20-90x10^6	22-90x10^6
Sperm Concentration		62.1x10^6	12.5x10^6	49x10^6	48 .7x10^6
Semen volume		3.9(3-5ml)	3.7(2.5-5ml)	3.2(1.5-5ml)	4.3 (2.5- 6ml)
Liquefaction time		17.5(10-25)min	17 (10-25) min	18.5 (10-30) min	20 (10-35)min
Total motile sperm		(50-80%) 67.5%	35-66% (50.1%)	(5-15%) 10.5%	54.8% (35-80) %
Abnormal forms		28.5%(15-35 %)	35.5%(25-50)	35% (30-45%)	91.7% (85-100%)
Period of infertility		-----------------	3-12	3-11	3-11

Ultrastructure Study of Sperms:

Using image analysis system for conducting sperm morphology parameters assessment using high magnification power showed that diameters of sperm head length in the normal and infertile groups reached (4.41± 0.41µm), (4.21± 0.50 µm) in Oligospermia, (3.58±0.43 µm) asthenozoospermia and (4.35 ± 1.66µm) in teratozoospermia. While, head width reached (2.54 ± 0.28 µm) in normal group, (2.35 ± 0.24 µm) in oligozoospermia, (2.40 ± 0.17 µm) in asthenozoospermia and (2.38 ± 0.35 µm) In teratozoospermia. Mead diameter of middle piece of the sperm was (5.99± 0.52 µm) in normal group while reached (4.8 ± 0.60 µm) in oligozoospermia, (4.21 ± 0.71 µm) in asthenozoospermia and (1.63 ± 0.70 µm) in teratozoospermia. Regarding to tail length of sperm, the mean tail of the sperm was (45.31 ± 5.45µm) in normal group. While reached (45.30 ± 0.99 µm) in oligozoospermia, (45.05 ± 0.85µm) in asthenozoospermia and (40.89 ± 5.17 µm) in teratozoospermia. Finally, the mean Acrosomal length of the sperm was (2.49 ± 1.14 µm) in normal group and reached (2.31± 0.38 µm) in oligozoospermia, (2.22 ± 0.16µm) in asthenozoospermia and (1.23 ± 1.31 µm) in teratozoospermia as shown in table (2) and plate (1-4).

Table 2: Sperm ultra-structures diameters (µm) in both normal fertile and infertile groups.

Sperm Structures	Control	Oligospermia	Asthenospermia	Teratospermia
Head Length	4.41± 0.41	4.21± 0.50	3.58±0.43	4.35 ± 1.66
Head Width	2.54 ± 0.28	2.35 ± 0.24	2.40 ± 0.17	2.38 ± 0.35
Middle Piece	5.99± 0.52	4.8 ± 0.60	4.21 ± 0.71	1.63 ± 0.70
Tail Length	45.31 ± 5.45	45.30 ± 0.99	45.05 ± 0.85	40.89 ± 5.17
Acrosomal Length	2.49 ± 1.14	2.31± 0.38	2.22 ± 0.16	1.23 ± 1.31

Data expressed as: Mead ± Standard Deviation.

Sperm DNA Content:

The acquired images of stained sperm with Methyl Green Pyronin for assessment of DNA was analyzed using special software program (Quantimet 500 +). The obtained data revealed that the measurements of optical densities of DNA from normal reached (1.03 ± 0.16 µm), oligozoospermia (0.90 ± 0.04), asthenozoospermia (1.00 ± 0.17) and teratozoospermia (0.86 ± 0.08) as shown in figure (3) and plate (1-4).

Table 3: Sperm DNA concentration in both fertile and infertile groups.

Parameter	Control	oligospermia	Asthenospermia	Teratospermia
Mean ± SD	1.03 ± 0.16	1.00ns ± 0.17	0.90ns ± 0.04	0.86* ± 0.08
%of change	---	-3.00%	-1.44%	-19.76%
95% C.L.	0.91 – 1.14	0.87 – 1.12	0.86 – 0.93	0.79 – 0.92
Range	0.93 – 1.45	0.65 – 1.32	0.81-0.97	0.69 – 0.97

Data expressed as: Mead ± Standard Deviation, % = percentage of change from control,
95% C.L. = confidence limits, n.s = non-significant and *= significant.

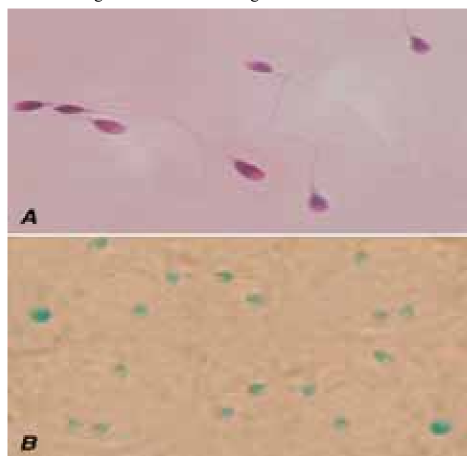

Plate 1: Photomicrographs of semen sample of normal group. A-The distribution of normal spermatozoa and there are absence of nucleated cells (H&E X 1000). B- The normal distribution of nucleic acid content in the sperm heads (Methyl green pyronin X 1000).

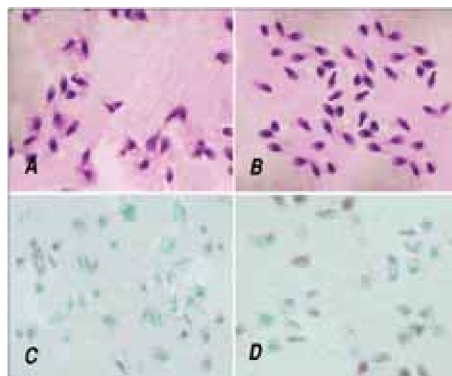

Plate 2: Photomicrographs of semen samples of oligospermic group. A-B- The distribution of spermatozoa (H&E X 1000). C- The distribution of faintly stained nucleic acid content in the sperm heads. (Methyl green pyronin X 1000). D-The distribution of nucleic acid content in the sperm heads (Methyl green pyronin X 1000).

Semen Parameters and Fertilization Rate:

In this study, 302 oocytes were obtained from oligospermia group (99 oocytes), asthenospermia group (105 oocytes) and teratospermia group (98 oocytes). When DNA integrity was measured by methyl green pyronin and sperms for ICSI were selected according to this criterion, the fertilization rate was reached (83.83 %, 86.66 % and 84.69 %) in Oligospermia, asthenospermia and teratospermia groups respectively as shown in table (4).

Embryo Quality:

The average percentage of embryo quality (grade A, B, C&D) in oligospermia, asthenospermia and teratospermia groups is summarized in Table 4. In this study oligospermia group (10) cases had 99 injected oocytes with 83.83 % fertilization rate and 27 embryos were transferred, six cases achieved positive chemical pregnancy (60%). While, asthenospermia group achieved 5 cases clinical pregnancies (50%) the injected Oocytes were (105) and fertilized oocytes (91) with 86.66 % fertilization rate and 27 embryos were transferred

5 cases achieved chemical pregnancy. The injected oocytes in the teratospermia group were (98), 83 oocytes with 84.69% out of them were fertilized and 29 embryos were transferred to achieve 5 cases chemical pregnancy (50%). There was no statistically significant difference in fertilization rate, implantation rate and clinical pregnancy rate between all groups as shown in Table (4).

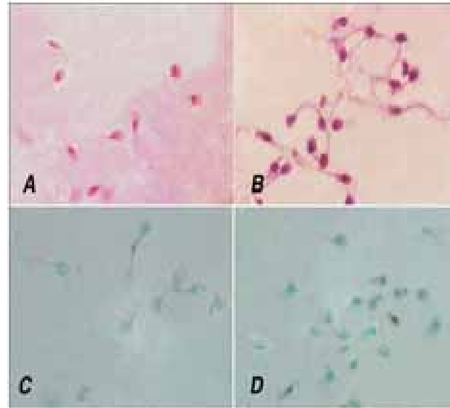

Plate 3: Photomicrographs of semen samples of asthenospermic group. A-B The distribution of spermatozoa (H&E X 1000). C- The distribution faintly stained of nucleic acid content in the sperm heads (Methyl green pyronin X 1000). D- The distribution of nucleic acid content in the sperm heads (Methyl green pyronin X 1000).

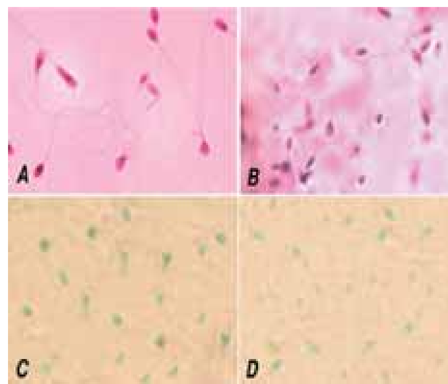

Plate 4: Photomicrographs of semen samples of Teratospermia group. A-B- The distribution of spermatozoa (H&E X 1000). C- The distribution of faintly stained nucleic acid content in the sperm heads. (Methyl green pyronin X 1000). D- The distribution of normal nucleic acid content in the sperm heads (Methyl green pyronin X 1000).

Table 4: Showing ICSI outcome and embryo quality fertilization rate and pregnancy.

Parameters	Oligospermia	Asthenospermia	Teratospermia
No. of Injected Oocytes	99	105	98
No of fertilized oocyte	83 (83.83%)	91 (86.66%)	83 (84.69%)
No. of Oocyte with 1PN	3	4	6
No. of Oocyte with 2PN	80	87	76
Oocytes arrested 2PN stage %	--	--	--
Fertilization Rate	83.83 %	86.66 %	84.69 %
GI (A) Embryos %	60 (75)%	55(63.2%)	35 (46%)
GII (B) Embryos %	10 (12.5)	15 (17.2%)	20 (26.3%)
GIII (C) Embryos %	10 (13.1)%	8 (9.1%)	5 (6.25%)
GIV (D) Embryos %	11 (14.4)%	9 (10.3%)	5 (6.25%)
No. Embryos Transferred	27	27	29
No. of Chemical Pregnancy	6	5	5

Discussion:

Semen analysis is the most common and informative laboratory technique for the investigation of male infertility despite its limited prognostic value in predicting the achievement of pregnancy. Sperm motility characteristic are important semen parameters that before the 1980s could be evaluated subjectively (Spiropoulos, 2001).

The conclusion of infertility or absolute infertility cannot be reached except in cases where DNA values are very low. This method can be used to choose cases for ICSI by DNA staining and proper statistical evaluation .the latter will be a non-biased method for evaluation. In the same time DNA studies should be carried further more to reach a better evaluation for infertility. It is now obvious that the evaluation of infertility is still very difficult to reach with routine tests.

According to spermiogram methods, the present study subjects were classified into normal, oligospermic, asthenospermic and teratospermic groups and sperms were stained with methyl green pyronin for assessment of DNA integrity and influence of selected embryos on the ICSI outcome. Some sperm may have a big amount of DNA still not motile but the main finding that DNA content is proportionally directed with fertility. Abnormality of the nuclear chromatin and DNA content are probably one of the causes of morphological aberration of the sperm head. (Larson *et al.* 2001, Ausió *et al.*, 2007 and De Sanctis *et al*., 2008).

Concerning the results of sperm ultrastructure in this study it was compatible with WHO (1999) criteria which recommended normal dimensions of head length, ranged from 4 µm to 5.5 µm and head width range from 2.5 µm to 3.5 µm. And also supported by (Kruger *et al.*, 2007) who found the length of the head in normal sperm ranged from 5 µm to 6 µm and width ranged from 2.5 µm to 3.5 µm. Also found the normal dimensions in these studies may be due to the difference in the type of staining used (Kruger, 2007) and also the method applied for measurement. In this study, we used a more accurate-computerized method (image analyzer). As a result of this, we determined the dimensions of different infertile groups exactly. Sperm motility depends on the flagellum, the organelle that develops the propulsive force for swimming (Kruger, 2007). So that, the mean length of the sperm tail may be used as an additional parameter for evaluation for motility and hence fertility (Leopardi *et al.,* 2005).

In this study, the length of the acrosome showed marked decrease which has no affect on its function and these findings supported by (Aziz, 2006; Bergqvist *et al.*, 2007 and Teijeiro, 2008) who proved that the length of the acrosome and subsequently its contents has no role in the acrosome reaction. It is better to evaluate a cromosomal function by enzymatic functions rather than by morphological measurements, this may not be applied to extreme values.

In conclusion it's clear that a classic semen analysis provides only incomplete information about the functional integrity of the spermatozoa. Furthermore, the interpretation of the results obtained from the conventional semen analysis, and the diagnosis reached, need to be reconsidered. The identification, and the treatment, of the infertile male will not make strides forward. Until better diagnostic and treatment modalities are developed, so we will continue to solve male infertility problems with assisted reproduction techniques, rather than curing the men.

REFERENCES

Al-Hasani, S., L.C. Demirel, B. Schöpper, M. Bals-pratsch, N. Nikolettos, W. Küpker, 1999. Preg-nancies achieved after frozen-thawed pronuclear oocytes obtained by intracytoplasmic sperm injection with spermatozoa extracted from frozen-thawed testicular tissues from non-obstructive azoospermic men. Hum. Reprod, 14(8): 2031-2035.

Aravindan, G.R., J. Bjordahl, L.K. Jost *et al.*, 1997. Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single- cell electrophoresis. Exp Cell Res, 236: 231-7.

Ausió, J., J.M. Eirín-López and L.J. Frehlick, 2007. Evolution of vertebrate chromosomal sperm proteins: implications for fertility and sperm Competition. Soc. Reprod. Fertil., 65: 63-79.

Aziz, D.M., 2006. Assessment of bovine sperm viability by MTT reduction assay. Anim.Reprod. Sci., 92 (1- 2): 1-8.

Banks, S., S.A. King, D.S. Irvine *et al.*, 2005. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. Reproduction, 129: 505-14.

Bancroft, J.D. and M. Gamble, 2002. Enzyme histochemistry and diagnostic application in theory and practice of histological techniques. Bancroft, J.D. and Gamble, M. (eds.) 5th ed., London, Edinburgh, new York, Philadelphia, Tronto, 595-620.

Bartoov, B., A. Berovitz, F. Eltes, 2001. Selection of sperm with normal nuclei to improve pregnancy rates with ICSI [letter]. N. Engl. J. Med., 345: 1067-8.

Bergqvist, A.S., J. Ballester, A. Johannisson, N. Lundeheim and Rodríguez-Martínez, 2007. Heparin and dermatan sulphate induced capacitation of frozen thawed bull spermatozoa, J. Repro. Fertil., 15(3): 225-232.

Bianchi, P.G., G.C. Manicardi, D. Bizzaro, *et al.*, 1993. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. Biol Reprod, 49: 1083-8.

Brewer, L.R., M. Corzett, R. Balhorn, 1999. Protamine induced condensation and decondensation of the same DNA molecule. Science, 286: 120-3.

De Jonge, C.J., 2000. Paternal contributions to embryogenesis. Reprod. Med. Rev., 203-214.

De Sanctis, V., D. Perera, M. Katz, M. Fortini and M.R. Gamberini, 2008. Spermatozoal DNA damage in patients with thalassaemia syndromes .Pediatr Endocrinol Rev., 6(1): 185-190.

Ebner, T., C. Yaman, M. Moser, M. Sommergruber, W. Pölz, G. Tews, 2001. Embryo fragmentation in vitro and its impact on treatment and pregnancy outcome. Fertil. Steril., 76(2): 281-285.

Erenpreiss, J., J. Bars, V. Lipatnikova *et al.*, 2001. Comparative study of cytochemical tests for sperm chromatin integrity. J. Androl., 22: 45-53.

Erenpreiss, J., S. Hlevicka, J. Zalkalns *et al.*, 2002. Effect of leukocytospermia on sperm DNA integrity: a negative effect in abnormal semen samples. J. Androl., 23: 717-23.

Evenson, D., L. Jost, D. Varner, 2000. Stallion sperm nuclear protamine -sh status and susceptibility to DNA denaturation are not strongly correlated. J. Reprod. Fert. Suppl., 56: 401-406.

Evenson, D.P., L.K. Jost, D. Marshall, 1999. Utility of the sperm chromatin assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod., 14: 1039-49.

Fischer, M.A., J. Willis, A. Zini, 2003. Human sperm DNA integrity: correlation with sperm cytoplasmic droplets. Urology., 61: 207-11.

Gatewood, J.M., G.R. Cook, R. Balhorn, *et al.*, 1987. Sequence-specific packaging of DNA in human sperm chromatin. Science, 236: 962-4.

Gineitis, A.A., I.A. Zalenskaya, P.M. Yau *et al.*, 2000. Human sperm telomere-binding complex involves histone H2B and secures telomere membrane attachment. J Cell Biol., 151: 1591-8.

Guzick, D.S., J.W. Overstreet, P. Factor-Litvak, *et al.,* 2001. Sperm morphology, motility, and concentration in infertile and fertile men. N. Engl. J. Med., 345: 1388-93.

Irvine, D.S., J.P. Twigg, E.L. Gordon, *et al.*, 2000. DNA integrity in human spermatozoa: relationshipswith semen quality. J. Androl., 21: 33-44.

Jarow, J.P., R.E. Budi, M. Dym, B.R. Zirkin, S. Noren and F.F. Marshall, 1985. Quantitative pathologic changes in the human testis after vasectomy. A control study. New Engi.J. Med., 313: 1252-1256.

Jeyendran, R.S., 2000. Interpretation of semen analysis results. A practical guide, 1 st ed, Cambridge University press: 1-50.

Joris, H., Z. Nagy and H. Van de Velde, 1998. Intracytoplasmic sperm injection: laboratory set-up and injection procedure. Hum. Reprod, 13(1): 76-86.

Kodama, H., R. Yamaguchi, J. Fukuda *et al.*, 1997. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. Fertil Steril, 68: 519-24.

Kosower, N.S., H. Katayose, R. Yanagimachi, 1992. Thiol-disulfide status and acridine orange fluorescence of mammalian sperm nuclei. J Androl., 13: 342-8.

Kruger, T.F. and K. Coetzee, 2007. The role of sperm morphology in assisted reproduction. Hum. Reprod. Update, 12(9): 565-576.

Larson, K.L., J.D. Brannian, N.P. Singh, J.A. Burbach, L.K. Jost, K.P. Hansen, D.O. Kroger and D.P. Evenson, 2001. Chromatin structure in globozoospermia case report. J. Androl., 22(3): 424-31.

Leopardi, P., P. Villani, E. Cordelli, E. Siniscalchi, E. Veschetti and R. Crebelli, 2005. Assessment of The in vivo genotoxicity of vanadate: analysis of micronuclei and DNA damage induced in mice by oral exposure. Toxicology Lett., 158(1): 39-49.

Mallidis, C., 2003. Necrospermia and chronic spinal cord injury. Fertil. Steril., 74: 221-227.

Mc Lachlan, R.I., H.W. Baker, G.N. Clarke, K.L. Harison, P.L. Matson and C.A. Holden, 2003. Semen analysis. Board of education of royal modern Reproductive medical practice.Patho.Androl., 35(1): 25 - 33.

Mortimer, D. and R. Menkveld, 2001. Sperm morphology assessment-historical perspectives and current opinions. J. Androl., 21: 22-192.

Oliva, R., 2006. Protamines and male infertility. Hum Reprod Update, 12: 417-35.

Potts, R.J., C.J. Newbury, G. Smith, *et al.*, 1999. Sperm chromatin damage associated with male smoking. Mutat Res., 423: 103-11.

Sailer, B.L., L.J. Sarkar, J.A. Bjordahl *et al.*, 1997. Effects of heat stress on mouse testicular cells and sperm chromatin structure. J. Androl., 18: 294-301.

Saleh, R.A., A. Agarwal, R.K. Sharma, *et al.*, 2003. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. Fertil Steril., 80: 1431-6.

Spano, M., J.P. Bonde, H.I. Hjollund *et al.*, 2000. Sperm chromatin damage impairs human fertility. Fertil Steril., 73: 43-50.

Sperm-cervical mucus interaction. 4th ed. New York: Cambridge University Press, 1999.

Spiropoulos, J., 2001. Computerized semen analysis (CASA): Effect of semen concentration and chamber depth on measurements. Arch. Of Androl., 46: 37-42.

Steger, K., K. Pauls, T. Klonisch *et al.*, 2000. Expression of protamine-1 and -2 mRNA during human spermiogenesis. Mol Hum Reprod., 6: 219-25.

Teijeiro, J.M., M.O. Cabada, P.E. Marini, 2008. Sperm binding glycoprotein (SBG) produces calcium and bicarbonate dependent alteration of acrosome morphology and protein tyrosine phosphorylation on boar sperm. J. Cell Biochem., 103(5): 1413-1423.

Van Steirteghem, A.C., Z. Nagy and H. Joris, 1993. High fertilization and implantation rates after intracytoplasmic sperm injection. Hum, Reprod, 8(7): 1061-1066.

Ward, W.S., D.S. Coffey, 1991. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol Reprod., 44: 569-74.

Ward, W.S., A.O. Zalensky, 1996. The unique, complex organization of the transcriptionally silent sperm chromatin. Crit Rev Eukaroyt Gene Expr., 6: 139-147.

World Health Organization, 1999. WHO Laboratory Manual for the examination of Human Semen and Semen-Cervical Mucus Interaction. 4th ed. Cambridge, United Kingdom: Cambridge University Press: 3-200

Zini, A., R. Bielecki, D. Phang, *et al.*, 2001. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. Fertil. Steril., 75: 674-7.

Zini, A., G. Defreitas, M. Freeman *et al.*, 2000. Varicocele is associated with the abnormal retention of cytoplasm by human spermatozoa. Fertil. Steril., 74: 461-4.

Zini, A. and J. Libman, 2006. Sperm DNA damage: clinical significance in the era of assisted reproduction. CMAJ, 175(5): 495-500.