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Eradication of DNA damaged sperm using swim-up and density gradient separation methods

KEYWORDS: Sperm wash, DNA damage, swim- up, density gradient separation, comet assay and flowcytometry.

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ABSTRACT ***INTRODUCTION:*** *Swim-up techniques for sperm separation may have detrimental effects on sperm DNA. We wished to determine whether the normal swim-up method with centrifugation used in our laboratory, which involves a centrifugation step, was harmful to sperm compared with swim-up without centrifugation.* ***METHODS:*** *Semen samples were obtained from patients undergoing IVF or andrology assessment. An aliquot was removed for fixation and subsequent DNA fragmentation determination. The remaining sample was divided into two equal parts, which was subjected to swim-up either with (normal swim-up) or without (direct-swim-up) centrifugation. Semen analysis was performed both before and after swim-up. DNA fragmentation, in spermatozoa previously fixed in 4% paraformaldehyde, was assessed by the terminal transferase-mediated DNA end labeling procedure (TUNEL). The percentage of spermatozoa with DNA damage after each swim-up technique was compared with that in the original semen sample.* ***RESULTS:*** *DNA damage was <5 % in most sample. No significant change in DNA fragmentation was observed between two swim-up procedures but significantly changes were observed between healthy men and infertile men.* ***CONCLUSIONS:*** *we conclude that our TUNEL technique based flowcytometry are useful to differentiate the live and damaged sperm with their percentage.*

Introduction

Infertility is one of the major problems for human being. Approximately 20% couples are suffering from infertility and they are trying to find suitable medicine in their marriage life *(Hull et al., 1985)*. According to statistics data both men and women are infertile, that is about 33% infertility problems are related to men, 33% to women and remaining are mixture of both male as well as female related infertility.

Male infertility refers to be deficient in of ability of a male to attain a pregnancy in a fertile female (University of Utah Health Sciences Center, 2010). A huge data are available over the past twenty years reveal that in approximately 30% of cases pathology is found in the man and another 20% both either man as well as woman are abnormal. Therefore, the male factors are partly responsible in about 50% of infertile couples *(Brugh et al., 2004; Hirsh 2003)*. There are many factors are responsible for male infertility such as sperm abnormalities, Varicocele, immunologic factors causing antisperm antibody formation, ejaculatory duct obstruction, hormonal inequality, azoospermia factor micro deletions and chromosomal aberrations and several environmental factors. While prevalence of infertility is maximum and approximately 50% of the infertility problems are largely or moderately due to a male factor *(World Health Organization, 1987)*, very few diagnostic tools are available to diagnose male fertility *(Jequier, 2004)*.

Semen quality is one of the most important indicator of male fertility semen quality such as Semen pH, Viscosity, Volume of the semen sample, Sperm motility, Liquefaction time, Sperm count, Sperm morphology and, White blood cell count in semen and Fructose level as per the respective lab standards that help or hinder conception. On the other hand, men with poor semen analysis results may go on to father children *(Essig et al., 2007)*. Volume of the semen is measured to shortlist the possibility of blockage or inflammation. Low semen volume is characteristic of obstruction of the ejaculatory duct or Congenital Bilateral Absence of the Vas Deferens (CBAVD) condition is also indicates that the seminal vesicles are undeveloped or poorly developed and high semen volume may indicate inflammation of accessory glands *(de la Taille A et al 1998; von Eckardstein S. et al 2000; Weiske WH et al 2000; Daudin M et al.2000)*. The total number of spermatozoa (sperm number) and the number of spermatozoa per unit volume of semen (sperm concentration) is related to both time to pregnancy and pregnancy rates, which are predictors to conception (Slama R et al 2002; Zinaman MJ et al.. 2000; Larsen L et al. 2000; Bonde JP et al 1998; WHO 1996). The normal ejaculates; the sperm number is evaluate of the capability of the testes which produce spermatozoa and the patency of the male tract while sperm concentration is not a specific measure of testicular *(Handelsman DJ et al. 1984;WHO1987; Andersen AG et al, 2000; Behre HM et al. 2000; MacLeod J et al,1979)*. The sperm concentration is also related to fertilization and pregnancy rates *(Eliasson R et al, 1979)*. It is based on the concentration and sperm number. The male infertility is differentiated into Oligozoospermia, Aspermia, Hypospermia, Azoospermia, Teratospermia and Asthenozoospermia.

Sperm motility and sperm morphology helps us to recognize the potentially fertilizing subpopulation of spermatozoa which may result in viable pregnancy. On the basis of the sperm motility the sperms are classified into progressive motility, nonprogressive motility and immotile sperms. The morphology of sperm identifies the structural defects of the sperm and it is classified into several defects such as head defects, mid piece defects, tail defects or multiple defects in a sperm.

The pH of Semen is one of the important and important parameter during analysis because abnormal pH of semen may indicate any infections or blockage of seminal vesicles. According WHO the normal pH range of semen is 7.2 to 8.0. Variation from this range indicates about an inflammation of the male accessory sex organs or chronic disease of the prostate gland and seminal vesicles. If the acidic prostatic secretion is takes place then seminal vesicles fluid will be alkaline. Acidic ejaculation may be associated with the blockage of the seminal vesicles *(de la Taille A et al 1998; von Eckardstein S et al 2000; Weiske WH et al 2000; Daudin M et al.2000)*, while Alkaline ejaculation is associated with infections that is harmful for invitro and invivo fertilization. In general a pH value outside the range is harmful to sperms morphology.

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The structure of normal sperm chromatin is essential for a correct transmission of paternal genetic information, and it is well documented that there is a negative correlation between defective sperm chromatin structure (DNA breaks) and fertility, in vitro *(Evenson et al., 1999; Spano et al., 2000)* and in vivo *(Larson et al., 2000; Evenson and Jost, 2000; Saleh et al., 2003; Larson-Cook et al., 2003; Gandini et al., 2004; Bungum et al., 2004; Virro et al., 2004; Check et al., 2005; Evenson and Wixon, 2006).* Although, 30% of patients seeking ART have high rates of sperm DNA breaks *(Bungum et al., 2004)*, very few clinics, so far, have implemented routine DNA integrity testing are staining with aniline blue, toludine blue, and chromomycin A3 can identify sperm chromatin packaging defects. The integrity of sperm DNA can be evaluated with the terminal deoxynuceotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL), comet, in-situ nick translation, or sperm chromatin structure assay (SCSA) techniques.

In the TUNEL technique, terminal deoxynucleotidyl transferase (TdT) incorporates dUTP biotinlyated deoxyuridine to 3'-OH at singleand double-strand DNA breaks to create a signal, which increases with the number of DNA breaks., DNA damage is quantified with the comet assay by measuring the displacement between the genetic material of the nucleus comet head and the resulting tail. In the SCSA technique, the extent of DNA denaturation following heat or acid treatment is determined by measuring the meta-chromatic shift in fluorescence after acridine orange staining. Sperm aneuploidies are usually detected using the fluorescence in situ hybridization (FISH) technique. In one of these tests, the sperm chromatin structure assay (SCSA) DNA fragmentation index (DFI) is used to get an estimate of DNA breaks, a parameter suggested as an independent predictor of fertility *(Evenson et al., 1999).* The etiology of sperm DNA damage is due to primary testicular or secondary (e.g. environmental) factors. The result of Sperm DNA damage is believed to be an aberrant protamine expression, excessive ROS (reactive oxygen species) generation and abortive apoptosis during spermatogenesis *(Sakkas et al., 2003; Aitken et al., 2009; Carrell et al., 2001; de Yebra et al,. 1993).* Endogenous and Exogenous ROS can induce sperm DNA damage in vitro, it confirms that ROS may play a role in the etiology of sperm DNA damage in infertile men *(Twigg et al.,1998; Aitken et al., 1998; Sawyer et al., 2003).* The association between semen ROS and sperm DNA damage is the basis for the use of antioxidants in the treatment of sperm DNA damage.

3. MATERIAL AND METHODS

3.1. Patient selection

Two hundred seventy four human semen samples from patients seeking assisted reproductive techniques (ART) treatments at the Arpit Infertility Research Center Allahabad, between January 2010 and May 2015, were used in this study. Informed consent for the use of spermatozoa was obtained.

All males aged between 21-50 years, clinically diagnosed with infertility or sub fertility including azoospermia, oligospermia, aspermia, asthenospermia, teratozoospermia or combined conditions were included as cases and males with proven fertility who passed all the criteria of the *(WHO (2010)* guideline dealing with spermiogram were included as controls. Other symptom containing men such as obesity, cardiovascular problem, HIV positive and Hepatitis (HBsAg) positive were excluded from the study.

3.2. Ethical clearance

The study was approved by the Institutional Human Ethical Committee (IHEC), numbered IHEC-UOM No. 54/Ph. D/ 2010-16. Informed consent to participate in the study was obtained from the subjects or their spouse.

3.3. Semen Collection for physical examination

After 3-5 days of ejaculatory abstinence the semen samples were collected in a sterile plastic container by the process of masturbation from the subjects (WHO, *2010).* Semen samples were collected in the laboratory room in a clean, dry, biologically inert container. The collected samples were allowed to liquefy at 37°C for 30 minutes and analyzed within one hour after collection. The semen samples were centrifuged at 3,000 rpm for 10 minutes and the seminal plasma was separated and stored under -80o C for further analysis. Macro and microscopic assessment of the semen was carried out to measure semen volume, sperm count, concentration, sperm motility, viability, morphology and leukocyte count according to the World Health Organization *(WHO, 2010)* Guidelines.

3.3.1. Physical examination

A general physical examination is an integral part of the evaluation of male infertility. In addition to the general physical examination, particular focus should be given to the genitalia including 1) examination of the penis; including the location of the urethral meatus; 2) palpation of the testes and measurement of their size; 3) presence and consistency of both the vasa and epididymis; 4) presence of a varicocele; 5) secondary sexual characteristics including body habitus, hair distribution and breast development; and 6) digital rectal exam. The diagnosis of congenital bilateral absence of the vasa deferentia (CBAVD) is established by physical examination. and 6) digital rectal exam. The diagnosis of congenital bilateral absence of the vasa deferentia (CBAVD) is established by physical examination.

3.3.2. Sperm Vitality:

Sperm vitality was estimated by using eosin and nigrosin staining to assess the membrane integrity of the cells. The percentage of viable cells normally exceeds that of motile cells.

3.3.3. Vitality test using eosin–nigrosin

This one-step staining technique uses nigrosin to increase the contrast between the ackground and the sperm heads, which makes them easier to discern. It also permits slides to be stored for re-evaluation and quality-control purposes **(Bjorndahl et al., 2003).**

A drop of 1% aqueous solution of eosin-Y and 10% aqueous solution of Nigrosin was taken in a tube. A drop of well mixed semen was added and mixed well using Pasteur pipette. A wet preparation of this mixture was observed under optical microscope. Dead sperms were stained red and live ones will remain unstained. At least 200 spermatozoa were counted and the incidence of live versus dead spermatozoa was estimated and expressed in percentage.

3.3.4. Estimating of Sperm concentration (sperm count) by cytometry

Dilution media was prepared by dissolving 50 g of sodium bicarbonate in 10ml 0f 40% formalin, 5ml of a saturated aqueous solution of gentian violet was added and make up to final volume of 1000 ml with distilled water. Around 10 - 15µl of semen sample was taken onto a cytometer and a cover slip was placed over it. The sample was allowed to settle down for about 5 minutes. High (>100 X 106. ml-1) density semen samples would require further dilution while low (<10 X 106. ml-1) density samples would require lesser dilutions. Number of spermatozoa in the central square of the Neubauer counting chamber was counted. The number of squares was examined for sperm enumeration will depend on the average number of spermatozoa present in a square. If there are <10 spermatozoa per squares should be examined, the occurrence of 10 – 40 spermatozoa per square would necessitate counting of 10 squares, 5 squares should be examined if there are >40 spermatozoa per square.

3.4. Sample collection and preparation

Samples were obtained by masturbation after two days of sexual abstinence and allowed to liquefy at 37°C for 10–20 min with 5% CO_2 in CO_2 incubator. Immediately after ejaculation neat semen sample were used to analyze the semen quality such as volume, motility and concentration according W.H.O. 2010. Each neat semen sample were aliquot 50 µl volume within one hours of ejaculation for Sperm DNA fragmentation (SDF) index assessment.

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3.4.1. Sperm wash by Swim up

0.5 ml semen sample were diluted with 1 ml of pre-warmed sperm preparation in EBSS medium (Earle's Balanced Salt Solutio), containing 0.1% human serum albumin (Cat No. E 2888, Sigma Chemical Co) and centrifuged at 200xg for 10 min. The supernatants were carefully discarded and and washed twice with same medium and carefully added 300 µl medium into pellet. The tube was put at 37 °C in CO2 incubator for one hrs and allows to motile the sperm to swim up from the pellet. After incubation sperm concentration, motility, viability, normal morphology and DNA integrity were estimated according to the WHO 2010 guidelines. Each SUP recovered semen sample was used for SDF assessment. To avoid the undesirable effects of iatrogenic DNA damage. All sperm samples were assessed for SDF immediately after sperm selection.

3.4.2. Sperm wash by density gradient method

Approximately 0.5 ml of sperm suspension was layered on a commercially available discontinuous two layer (40%-80%) gradient (PureCeption™, Cat. No.2040&2080, Sage) in a 14 ml tube. The tubes were centrifuged at 500 g for 20 min at room temperature. The upper and lower layers were carefully aspirated without disturbing the pellet. Finally, the pellet was resuspended in 1 ml of EBSS medium. Sperm concentration and motility were estimated in the recovered fractions according to *World Health Organization guidelines 2010.* All sperm samples were assessed for SDF immediately after sperm selection.

3.5. Sperm chromatin dispersion Test

Sperm DNA fragmentation (SDF) was determined by the Sperm chromatin dispersion (SCD) test. In this test we have used Halosperm kit (Halotech DNA, Madrid, Spain) Briefly, 25µl sperm cells from 1 X 107 spermatozoa /ml was taken in a vial containg low melting point agarose (Sigma, St Louis, MO, USA) and mixed. Pre-treated slides were placed onto a pre cooled metallic plate and a drop of the cell suspension was put onto the slide and covered with a glass coverslip and allowed to solidify at 4 °C for 10 min. The coverslip was removed carefully and the slide was horizontally placed in of the lysis solution provided in the kit. Finally, the slides were washed in distilled water and dehydrated in sequential baths of 70 to 100% ethanol then stained with propidium iodide (2 µg/ ml; Sigma) in Vectashield (Vector Laboratories, Burlingame, CA, USA). At that point, the samples could either be immediately analysed or stored at room temperature in the dark until needed. The SDF was established as the percentage of fragmented sperm cells in a semen sample. The SDF-d was defined as the percentage of degraded sperm cells in a semen sample. Both the SDF and the Sperm chromatin dispersion & Degradation (SDF-d) were calculated.

3.6. DNA damage analysis by Comet assay

The DNA fragmentation in spermatozoa was assessed by the alkaline comet assay as described by *Singh et al. (1988)* with slightly modifications. Briefly, the sperms were collected in sterile phosphate buffered saline (pH 7.4) and centrifuged. Sperm density was kept constant by appropriate dilution in order to maintain the uniform distribution of the spermatozoa during electrophoresis. The sperm suspension was mixed with an equal volume of 0.8% low melting agarose (Cat No. A 9414, Sigma Chemical Co, USA) and layered on a slide pre-coated with 1% normal agarose (Cat No. 9539, Sigma Chemical Co, USA). A third layer of agarose was layered over the second layer and incubate overnight in lysing solution (2.5M NaCl, 100 mM disodium EDTA, 10 mM Trizma base, pH 10.1% Triton X-100, 10mM GSH, and 100 ᴍM heparin) under alkaline conditions (pH 10) at 4°C. After sperm DNA unwinding in the electrophoresis buffer (300mM NaOH, 1mM EDTA for 20 minutes, electrophoresis was carried out at 25V for 20 min. The slides were neutralized in 0.4M Tris HCl buffer for 15 minutes. Then the slides were immersed in chilled absolute alcohol for 30 minutes for dehydration and then dry for until staining. DNA was stained with SYBR Green I (Molecular Probes, Leiden, The Netherlands) at a 1 : 3000 dilution in Vectashield medium (Vector Laboratories). At that point, the samples could be immediately analyzed or put at room temperature in the dark until further used. The frequency of sperm cells with SDF was established and Cells were classified as undamaged, ssDD or dsDD cells according to the comet assay's principle that the size of the tail and/or the density of DNA in the tail positively correlate with the extent of DNA.

3.7. Terminal deoxy nucelotidyl transferase mediated nick end labeling assay:

The assay was described according to standard protocol *(Piqueras et al., 1996)* with slightly modification. 50µl of semen sample was placed on a poly-L-lysine coated slide and put at room temp for dry. The cells were fixed in 4% paraformaldehyde solution for 30 minutes. Then 30 minutes (on ice) treated with 0.2% triton-X 100 for permeabilization of the sperm membranes and then washed three times with PBS. The sperm cells were incubated in terminal deoxynucleotide transferase (TdT) and nucleotide mix (Apoalert DNA fragmentation assay kit, Cat No. 630108, Clontech, USA) for 1 hour at 37ºC in a humidified incubator. The cells were washed thrice with PBS and counterstained with Propidium Iodide solution (10µg/ml). TUNEL positive cells showed a strong nuclear green fluorescence with fluorescence microscope (Imager-A1, Zeiss, Germany) equipped with a 490 nm excitation filter. In another experiment DNA damage was expressed as percentage of TUNEL positive spermatozoa through Flow cytometric analysis of sterile and fertile person. The sperm 5×105 ml-1 in each tube. Briefly, the cells were washed twice with cold PBS. The cell pellets were suspended in 1 ml of ice-cold ethanol for 1 h at 4 °C and centrifuged at 250 g for 5 min. The pellets were again washed twice with cold PBS and resuspended in 100 µl RNase (5mg ml-1) for 30 min at 37 °C. Thereafter, 300 µl propidium iodide (50 µg ml−1) was added and the mixture was incubated on ice for 1 h. Finally, the cells were analyzed on flow cytometer FACS CALIBER (Becton Dickinson).

3.5. Statistical analysis

The obtained data were subjected to statistical analysis using statistical software SPSS version 16.0. Data were expressed as Mean± Standard deviation. Student t-test, one way analysis of variance (ANOVA) was done to compare means. Statistical significance was analyzed by Chi-square test. Pearson correlation was performed to assess the linear relationship between semen parameters.

4. Results

4.1. Patient selection, Sample collection and physical examination

The age distribution of the participants in both infertile and control males is shown in Table 1and 2. Of the 274 infertile and clinically diagnosed with infertility or sub fertility including azoospermia, oligospermia, aspermia, asthenospermia, teratozoospermia or combined conditions were included as cases and males with proven fertility who passed all the criteria of the *WHO (2010)* guideline dealing with spermiogram were included as controls.

Table 1: Age-wise distribution of 274 Infertile men and 130 controls (n= number of study subjects).

Conditions	21-30 Years		31-40 Years		41-50 Years	
	No.	%	No.	%	No.	%
Infertile (n=274)	61	22.3	191	69.7	22	8.3
Control (n=130)	42	32.3	74	56.9	14	10.8

Table 2 : Age-wise distribution of different infertility among infertile group (n=274)

Conditions	Age group in years						Total
	21-30y		31-40y		41-50y		
	No.	%	No.	%	No.	%	
	1	0.36	11	4	2	0.7	
Azoospermia	30	10.9	58	21.2	10	3.6	98
Oligozoospermia	5	1.3	12	4.4	3	1.1	20

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Asthenozoospermia	5	1.3	20	7.2	6	2.2	31
Oliogasthenozoos- spermia	10	3.6	25	9.1	6	2.2	41
Oliogasthenteratonecrozoo spermia	10	3.6	38	13.8	-	-	48
Oliogasthenonecrozoosper mia	3	1.1	12	4.4	2	0.7	17
Oligoasthenoteratoz- oospermia	1	0.36	3	1.1	1	0.36	5

4.2. Physical examination

Both groups normal as well as infertile patient's sample were statically observed the physical parameter such as coital frequency, body mass index, seminal volume, sperm count, total sperm count , sperm motility and liquefaction etc.**(in Table 3, 4 and 5).**

Table 3: Mean and standard deviation of spermiogram among both groups. (CF=Coital Frequency, BMI= Body mass index, SV=Seminal volume, SC=Sperm count, TSC= Total sperm count, TSM= Total sperm motility, Liq= Liquefaction, n= number of study subjects).

	Groups	n	Mean± Std. Deviation	
Age	Infertile	274	34.15	4.87
	Normal	130	33.43	5.61
C.F.	Infertile	274	2.71	1.29
	Normal	130	2.35	1.37
BMI	Infertile	274	25.26	4.26
	Normal	130	25.16	4.25
SV	Infertile	274	1.56	0.90
	Normal	130	2.01	0.73
SC	Infertile	274	9.44	15.72
	Normal	130	59.42	22.01
TSC	Infertile	274	13.61	22.25
	Normal	130	118.68	63.76
TSM	Infertile	274	9.59	14.46
	Normal	130	54.41	12.25
Morphology	Infertile	274	4.50	6.74
	Normal	130	17.55	11.03
Viability	Infertile	274	27.82	28.98
	Normal	130	72.05	10.55
pH	Infertile	274	8.29	0.13
	Normal	130	7.74	0.32
Pus Cell	Infertile	262	2.15	2.92
	Normal	130	3.19	4.34
Liq. Time	Infertile	274	24.63	13.68
	Normal	130	27.23	11.95

Table 4: Independent t-test between spermiogram in infertile and control groups

(CF=Coital Frequency, BMI= Body mass index ,SV= Seminal volume, SC=Sperm count,
TSC= Total sperm count ,TSM= Total sperm motility, Liq= Liquefaction, CI=Confidence
Interval).

	Test for equality of variance		t- test for Equality of Means 95% CI				
	F	Sig	t	Df	Sig(2tailed)	Lower	Uper
Age	2.192	.140	1.318	402	.188	-.353	1.791
C.F.	3.305	.070	2.539	402	.011*	.081	0.635
BMI	0.142	.706	0.224	402	.823	-.7905	.9938
SV	9.029	.003	-4.939	402	.0001*	-.6251	-.2692
SC	33.039	.0001	-26.100	402	.0001*	-53.746	-46.216
TSC	131.212	.0001	-24.354	402	.0001*	-113.551	-96.588
TSM	2.461	.117	-30.513	402	.0001*	-47.700	-41.926
Morphology	35.350	.0001	-14.646	402	.0001*	-14.793	-11.292
Viability	416.618	.0001	-16.869	402	.0001*	-49.391	-39.081
pH	17.233	.0001	-.734	402	0.464	-.4404	.2010

*Significant at the P<0.05

Table 5: Pearson correlation between age and quantitative semen parameters among cases and controls.

(NS=non significant, r= Correlation coefficient, *. Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed).

		C.F.	BMI	SV	SC	TSC	TSM	Morphology	Viability	pH	pus cell	Liq Time
Age	r	-.073	0.151**	0.059	-.057	-.024	-.076	-.025	.022	.060	.018	-.049
C.F	r		0.023	-.004	-.113*	-.131**	-.101*	-.029	-.086	0.009	-.077	0.016
BMI	r			0.052	-.041	-.021	-.018	0.064	0.015	0.006	-.054	-.016
SV	r				0.152**	0.395**	.202**	0.185**	0.204**	-.098	0.054	0.081
SC	r					.883**	.817**	0.588**	0.753**	-.065	0.089	0.100*
TSC	r						0.751**	0.569**	0.666**	-.060	0.095	0.116*
TSM	r							0.653**	0.823**	-.075	0.091	0.126*
Morphology	r								0.681**	-.062	.130**	0.169**
Viability	r									-.092	.151**	0.096
pH	r										-.006	-.087
PusCell	r											0.004

4.3. Eeosin–nigrosin staing for viability test

Nigrosin to increase the contrast between the background and the sperm heads, which makes them easier to distinguish. **(Fig. 1)**

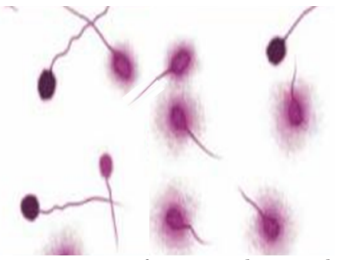

Fig. 1. Eeosin–nigrosin staing of sperm to distinguish the live and dead

4.4. Sperm chromatin dispersion Test

Semen sample were taken and wash with different technique such as

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SUP, density gradient technique used for Sperm DNA fragmentation assessment after sperm selection. Sperm DNA fragmentation (SDF) was determined by the Sperm chromatin dispersion (SCD) test. In this test we have used Halosperm kit (Halotech DNA, Madrid, Spain).

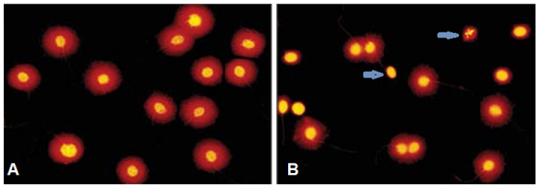


Fig. 2. SDF as determined by the SCD test. (A) Semen sample showing healthy men (B) Sperm cells with fragmented DNA in the microscope field.

4.5.Comet assay DNA damage

The DNA fragmentation in spermatozoa was assessed by the alkaline comet assay as described by Singh et al. (1988) with slightly modifications.

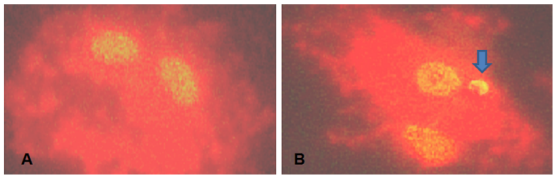
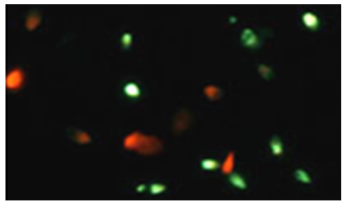


Fig.3. Comet assay. (A) Semen sample showing healthy men (B) Sperm cells with fragmented DNA .

4.6. Terminal deoxy nucelotidyl transferase mediated nick end labeling assay:

The assay was described according to standard protocol (Piqueras et al., 1996) with slightly modification. TUNEL assay is ananother experiment DNA damage was expressed as percentage of TUNEL positive spermatozoa through Flow cytometric analysis of the healthy men has approximately 00% and unhealthy men has 53.7% DNA damaged sperm by using the gating in flowcytometry analysis.



(C)

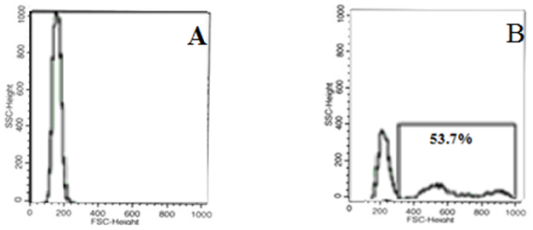


Fig.3. TUNEL assay. (A) Semen sample showing healthy men (B) Semen sample unhealthy men (fragmented DNA) and (C) Microscopic image.

Discussion

Infertility is a common upcoming problem wherein approximately 8% of men of reproductive age seek medical attention for infertility problems. Of these, up to 10% are with reversible causes affecting their fertility potential; varicocele represents 35% of these cases *(Esteves et al., 2011).* As such, the male partner must be systematically evaluated in every investigation of an infertile couple (Esteveset al., *2011).* Because 80% of couples are able to achieve pregnancy within the first year of attempting, a couple should only be diagnosed as infertile after one year of regular sexual activity without using a contraceptive method. Investigation is initiated earlier when risk factors are present, including advanced maternal (>35 years) or paternal age (>45 years), a history of urogenital surgery, cancer, cryptorchidism, varicocele, orchitis, use of gonadotoxins or genital infections, etc. *(Esteveset al., 2011).* The Andrologist is responsible for diagnosing, counseling and treating the underlying cause whenever possible. When there is no specific treatment he/she is still responsible for referring the patient to specialized assisted reproductive techniques (ART) center or, if the Andrologist is a member of an ART center's multi-professional team, for extracting the male gamete from the testicle or epididymis *(Esteveset al.,2011).*

For healthy young couples, the probability of achieving pregnancy per reproductive cycle is approximately 20 to 25%. The cumulative probabilities of conception are 60% within the first 6 months, 84% within the first year and 92% within the second year of fertility-focused sexual activity *(Kamel, 2010).* Infertility is a common clinical problem affecting 13 to 15% of couples worldwide *(WHO, 1984).* The prevalence varies throughout different countries, being higher in the underdeveloped nations where limited resources for diagnosis and treatment exist *(Cates et al.1985).* In the United Kingdom, infertility is believed to affect one in six couples *(Zargar et al.,1997).* According to *Kamel et al.,(2010)*, it should be regarded as a public health problem, as it affects not only the health care system but also the social environment *(Kamel, 2010).*

In the present study we detected a decrease in semen volume among infertile subgroups evaluated. Indeed, published reports showed a decrease in semen volume with ageing *(Ford Ng et al., 2004).* In the studies where the analyses were adjusted for the period of abstinence there was a decrease in semen volume of 3–22% *(Ford et al., 2000).* In our present study, sperm count was found to be significantly decreased in infertile males when compared with the controls and the negative correlation was also observed between age, BMI and total sperm count but not significant **(Table 2 and Table. 3).** This finding reveals that our data accord with the previous studies on the effect of age and BMI on male fertility potential. *Jensen et al., (2004)* reported a higher prevalence of oligozoospermia in overweight and obese men compared with normal-weight men (24.4% vs. 21.7%). Kort et al.. (2006) found that BMI correlated negatively with the total number of normal spermatozoa. *Najafi et al., (2012)* showed the similar result in Mysorepopulation. Central obesity in particular appears to be associated with a decrease in circulating androgen levels proportional to the degree of obesity. Data on the effect of age factor on sperm count accords with the similar results obtained in previous studies *(Eskenaziet al., 2006).* The main reason for non-significant values obtained for these factors in the present study could be due to a randomized sampling rather than a purposeful sampling.

In the present study sperm motility tended to decrease with age, indeed most studies have found a decrease in sperm motility with increasing age *(Ng et al.,2004).* Those studies that adjusted the results for the duration of abstinence reported statistically significant effects, such as negative linear relationships and decreases in motility of 0.17–0.6% for each year of age *(Kidd et al., 2001).* Thus, the present study supports the conclusion based on the data from most others, that there is consistent evidence for a decrease in sperm motility with increasing age although this correlation is not significant.

Present investigation showed that semen samples containing higher percentage of viable sperms were mostly with normal physical profile (Normozoospermia) where as abnormal semen samples were with poor viable sperms. Thus, viability of sperm may be considered as an authentic and handy tool to assess male fertilizing potential especially when facility for other sophisticated techniques if not available. Sperm morphology is a good indicator of the status of the germinal epithelium *(Bujan et al.,1996).* Degenerative changes

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in the germinal epithelium may be due to ageing which may affect spermatogenesis and thus sperm morphology. The results of our study clearly demonstrate that there is a significant increase in the frequency of sperm morphological defects among infertile males when compared with the control group **(Tables 4 and 5).**

On the contrary DNA damage has also been reported to arise during sperm processing. The morphology of the sperm as well as live or dead can be seen with Eeosin–nigrosin staing (Fig 1). Previous studies have shown *(Gosálvez et al., 2009).* After their isolation by density gradient centrifugation may lead to an increase in the levels of sperm DNA fragmentation. It also permits slides to be stored for re-evaluation and quality-control purposes *(Bjorndahl et al., 2003).* On the other hand, although density-gradient centrifugation was comparable to swim-up technique in recovering spermatozoa with enhanced motility, spermatozoa recovered after swim-up were found to possess higher DNA integrity *(Zini A et al., 2000).* A subsequent study which analyzed the influence of initial semen quality on sperm DNA integrity following semen processing also supported this contention, concluding that sperm processed by swim-up had superior DNA integrity *(Zini A et al., 2000).* Semen processing by density gradient centrifugation was not found to improve sperm apoptotic deoxyribonucleic acid fragmentation rates, leading the authors to suggest the use of other semen processing techniques in patients with underlying DNA fragmentation *(Stevanato J et al., 2008).*

In our study, we found no difference in the incidence of TUNEL positive sperm in the supernatant fractions of the various techniques, in all the samples, suggesting that all the three are comparable in yielding a population of sperm with low DNA damage. It has been reported that although discontinuous gradient eliminates morphologically abnormal sperm and swim-up treatment decreases DFI and HDS of spermatozoa, both methods are effective for embryo development *(Hashimoto Set al., 2008).* The first study to compare the effects of gradient-density centrifugation and swim-up techniques on sperm apoptosis using flow cytometry also suggested that both the sperm preparation methods allow obtaining a sperm population with low percentage of apoptotic sperm *(Ricci G et al.,2009).*

In summary, our results suggest that swim-up separation of motile spermatozoa from normal semen, either with or without centrifugation, does not increase the level of DNA damage and linear regression analysis of the data in this study indicated that there was a significant relationship between DNA fragmentations. The results of our study support the above findings.

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