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CASA derived human sperm abnormalities: correlation with chromatin packing and DNA fragmentation

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Abstract
Purpose The present study was undertaken to evaluate the effects of morphokinetic abnormalities of human spermatozoa on chromatin packing and DNA integrity and possible beneficial effects of sperm selection in ICSI.
Methods Semen samples from 1002 patients were analysed for morphology and motility using CASA. Protamine status and DNA fragmentation were analysed by chromomycin A3 staining and sperm chromatin dispersion assay respectively.
Results Sperms with elongated, thin, round, pyri, amorphous, micro and macro forms were significantly higher in teratozoospermic and oligoasthenoteratozoospermic groups. Significant difference in chromatin packing and DNA fragmentation index was observed in these abnormal groups compared with normal. Similarly significant correlation was also seen between abnormal motility parameters and DNA fragmentation index in asthenozoospermic group compared with normal.
Conclusions Specific abnormal morphological forms have higher incidence of chromatin packing abnormalities and DNA fragmentation. Using these sperms in ICSI might have an impact on fertilization, embryo development and abortion rates. These can be selectively avoided during ICSI procedure to improve ART outcome.

Keywords Male infertility · CASA · Motility · Chromatin packing · DNA fragmentation

Capsule Sperm morphological abnormalities as predictive indicator for abnormal chromatin and DNA integrity.

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Introduction

Human male infertility contributes significantly to the overall infertility problem. It is primarily evaluated by routine semen analysis which assesses the sperm count, motility and morphological classification [1]. Various morphological abnormalities of sperm head and morphometric parameters are often associated with infertility and to a large extent influence the fertilization cates and pregnancy outcome [2–4]. Traditionally, these parameters were routinely evaluated by light microscopy, but the drawbacks of this methodology such as subjectivity and high interobserver variability makes interpretation unreliable [5]. With the introduction of CASA (computer assisted semen analysis) assessment of the morphometric and morphological characteristics of spermatozoa is now more accurate, which until now was not possible by routine light microscopic analysis [6, 7]. In addition, this method also ensures improved accuracy in terms of better objectivity and precision compared to routine light microscopic evaluation.

Recently assays for sperm nuclear DNA (Deoxyribonucleic acid) fragmentation were developed and showed a strong positive correlation with sperm fertility status apart from the conventional semen morphology [8, 9]. Further, any subtle changes in sperm chromatin condensation or alteration in sperm histone-protamine ratio necessary for chromatin packing may directly reflect in changes in head

morphology and morphometry [10]. Experimental studies have demonstrated a positive correlation between abnormal sperm head morphology and DNA damage and chromatin integrity, but such relationship in infertile patients was not extensively studied [11–13]. To our knowledge, this is one of the few studies which compared and analyzed retrospectively, the correlation of sperm head morphology parameters assessed by CASA with DNA fragmentation analysis and chromatin integrity in infertile patients.

Material and methods

All chemicals were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) unless stated otherwise.

Study design and ethical aspects

Male patients seeking infertility treatment at Krishna IVF Clinic, India during the period December 2009 and April 2012 were recruited (n=1002) in this retrospective study. Informed consent was taken from all the patients who participated in this study and approved by Institutional review board (IRB), Krishna IVF Clinic. All couples included in this study for infertility investigations had a minimum period of 1 year of unprotected intercourse. Male patients with history of medical treatment, drug or alcohol abuse, heavy cigarette smoking, hypergonadotrophic hypogonadism and leukocytospermia were excluded from this study. All semen samples were subjected to CASA and classified as normozoospermia (N) and abnormal groups such as teratozoospermia (T), asthenozoospermia (A) and oligoasthenoteratozoospermia (OAT). Asthenoteratozooapermia (AT) and oligoteratozoospermia (OT) groups were included in OAT group.

Semen analysis

Semen samples were collected after a recommended sexual abstinence period of 2-5 days by masturbation. A part of semen sample was subjected to routine assessment to determine semen parameters such as volume, count, motility and morphology using CASA according to the Kruger strict criteria [14] and the rest of the sample was utilized for DNA fragmentation analysis and chromatin packing staining methods respectively. Sperm samples with concentration $\geq 20 \times 10^6$ /mL, motility ≥50 % and normal morphology ≥14 % were regarded as "normal" specimens.

Preparation of semen smear and staining method

All semen samples were subjected for staining procedure after complete liquefaction. Depending on the concentration, the volume of the sample was adjusted (ranging from 3-7 μl) for the smear preparation which results in 2-10 spermatozoa per field viewed using 100x oil immersion during morphometric analysis. The air dried smears were fixed in fixative solution and stained with Diff Quick stain as previously described [15].

Morphology analysis by CASA

Sperm count, motility and morphology parameters were included for the evaluation and analyzed using CASA (Sperm Class Analyzer (SCA), Version: 4.2; Microptic S.L., Barcelona, Spain and Microscope Nikon, Japan). At least 100 stained spermatozoa were analyzed using x100 oil immersion objective in bright field optics. Spermatozoa with borderline morphologies were considered as abnormal. A total of seven morphological variations of head (tapered, thin, round, pyri, amorphous, micro and macro) were assessed. CASA settings were adjusted before analyzing the semen samples as reported earlier by Rama raju et al. Briefly, the number of images captured per field was 25, and the number of images captures per second was also 25. Scale was calibrated at 10. Counting chamber selected was Makler (Sefi Medical Instruments, Haifa, Israel). Type of capture was manual. Cell size range (low), 2 mm^2, cell size range (high), 60 mm^2 [16].

DNA fragmentation analysis by SCD (Sperm Chromatin Dispersion) assay

The method used in this study was reported by Rama raju et al. Briefly, semen samples were diluted with phosphate buffer saline (PBS) to obtain a sperm concentration that ranged between 5 and 10 million. The suspensions were mixed with 1 % agarose (to get a final agarose concentration of 0.7 %) at 37 °C. Aliquots of 25 μl of the above mixture were pipetted onto a pre-coated agarose glass slide (0.65 %) and covered with glass coverslip (22×22 mm). The slides were then allowed to solidify for 5 min at 4 °C. Coverslips were gently removed, and the glass slides were immersed horizontally in a tray containing freshly prepared denaturation solution (0.08 N Hydrochloric acid [HCl]) for 7 min at room temperature. Denaturation was then stopped by transferring the slides to another tray containing fresh 10 ml lysis buffer [0.4 m Tris, 0.4 m DTT (Dithiothreitol), 50 mm EDTA (Ethylenediaminetetraacetic acid), 0.3 % SDS (Sodium dodecyl sulfate) and 1 % Triton X-100] and incubated for 25 min at room temperature. Slides were then thoroughly washed with distilled water, followed by dehydration for 2 min in each of 70 %, 90 % and 100 % ethanol and subsequently air dried. The dehydrated slides were stained with Giemsa and observed under bright field microscopy for halos. The degree of DNA dispersion was assessed by observing the relative halo size under bright field

microscopy (Zeiss Axioskope 2 plus; Carl Zeiss, Gottingen, Germany). A minimum of 500 spermatozoa per sample were evaluated. In brief, four different dispersion patterns based on halo size were observed under bright field microscopy; (i) sperm nuclei with big halo, (ii) sperm nuclei with medium sized halo, (iii) sperm nuclei with very small halo and (iv) sperm nuclei without halo. Sperm with large and medium sized halos are considered to be normal or non-fragmented and sperm with small sized halo or no halo are considered to have significant DNA fragmentation [16].

Chromomycin A3 (CMA3) staining

The method used in this study was reported by Bianchi et al. Briefly, Semen samples were washed twice with PBS at 3000 rpm for 2 min. Semen smears were prepared, air dried and fixed in Carnoy's fixative at 4 °C for 5 min. For staining, 20 μl of CMA3 stain was pipetted onto the slide, covered with glass coverslip (22×22 mm) and incubated at room temperature for 20 min. Slides were rinsed in PBS and mounted with buffered glycerol [17]. Sperm head staining pattern was observed under fluorescent microscope with an oil immersion objective(Zeiss microscope, Axioskope 2 plus; Carl Zeiss, Gottingen, Germany) at 63 x magnification with appropriate filters (460-470 nm). For each slide a total of 500 spermatozoa were randomly evaluated. CMA3 tends to bind to protamine deficient sperm and makes them appear bright yellow (abnormal chromatin packing), while sperm with intact protamine does not bind CMA3 and exhibits light or dull yellow coloured fluorescence (normal chromatin packing).

Aniline blue (AB) staining

The method used in this study was reported by Henkel et al. Briefly, semen smears were prepared by spreading a drop of washed semen onto the glass slide and allowed to air dry. All smears were fixed in 3 % buffered glutaraldehyde for 30 min and stained with 5 % aqueous aniline blue and mixed with 4 % acetic acid (pH 3.5) for 7 min [18]. Spermatozoa were observed under oil immersion with bright field microscopy (Axioskope 2 plus; Carl Zeiss, Gottingen, Germany) at 63x magnification. Two types of staining patterns were observed, namely sperm head with dark blue (presence of histones) and light or partial blue staining (absence of histones).

Statistical analysis

Statistical analysis was performed using Sigma stat 3.1 software (Systat software Asia Pacific Limited, Bangalore, India). Semen parameters, DNA fragmentation analysis and chromatin packing abnormalities in normal and different abnormal groups were compared using Student's *t*-test. Values are expressed as mean±SD. In order to detect the statistically significant difference for sperm morphological, sperm kinematic parameters, DNA fragmentation and chromatin packing abnormalities between normal and abnormal groups, a sample size of 50 patients are required to have a study power of >95 % and alpha of <0.05. The present study is a retrospective study from December 2009 to April 2012 and a total of 1002 patient data were analyzed, resulting in a much higher power than statistically required. A p value of <0.05 or less is considered as statistically significant.

Results

There was no significant difference in the mean age, marital life, body mass index (BMI), semen volume, and abstinence period between normal and all abnormal groups (Table 1).

Table 2 shows the sperm morphological parameters between normal and abnormal groups. The percentage of normal morphology, head normal size and normal shapes

Table 1 Mean age, marital life, BMI, semen volume and abstinence period between normal and abnormal groups

Parameters	Normal group	Abnormal group			*P* value
	N (*n*=277)	T (*n*=385)	A (*n*=102)	OAT (*n*=238)	
Age	35.84±4.53	36.27±5.59	35.43±7.92	36.12±4.43	NS
ML	6.43±3.82	6.15±3.65	5.85±4.72	6.70±3.17	NS
BMI	26.85±3.44	26.80±3.73	27.10±3.50	27.33±4.02	NS
Semen volume	2.35±1.25	2.28±1.22	2.41±1.58	2.43±1.48	NS
Abstinence period	4.28±5.08	3.82±3.06	4.22±4.16	3.81±3.55	NS

values are expressed as±SD

N normozoospermia, *T* teratozoospermia, *A* asthenozoospermia, *OAT* oligoasthenoteratozoospermia, *ML* married life, *BMI* body mass index

NS not significant

Table 2 Comparison of sperm head morphology between normal and abnormal groups

Parameters	Normal group	Abnormal group		
	N	T	A	OAT
Normal morphology (%)	16.35±4.90	5.12±0.55*	16.10±1.96	3.86±2.89*
Abnormal morphology (%)	83.61±5.35	94.25±3.01*	83.57±1.95	96.10±2.89*
Head normal size (%)	73.49±25.62	57.33±24.78*	71.08±23.59	50.07±23.39*
Normal shape (%)	35.84±13.52	17.40±13.15*	34.43±12.59	10.18±13.52*
Micro heads (%)	8.63±11.32	17.11±17.60*	9.35±8.59	21.07±19.19*
Macro heads (%)	17.56±27.17	25.38±28.48*	19.28±25.15	28.79±26.58*
Tapered (%)	54.51±13.45	60.95±13.31*	54.67±12.70	62.23±15.82*
Thin (%)	1.92±4.08	8.92±10.02*	2.30±2.28	10.44±15.70*
Round (%)	1.28±1.67	3.38±2.67*	1.97±2.67	5.50±2.65*
Pyri (%)	0.45±0.98	1.95±2.70*	0.56±0.93	2.97±4.24*
Amorphous (%)	5.39±5.72	7.24±9.59*	6.03±7.30	8.56±7.76*
Acrosome normal (%)	66.44±21.49	52.88±25.61*	67.18±19.35	49.02±28.39*

values are expressed as±SD

* $P<0.05$ is considered as statistically significant when compared to normozoospermic group

were significantly more in normal group compared with teratozoospermic and oligoasthenoteratozoospermic groups ($P<0.05$), where as no significant difference was observed between asthenozoospermic and normal group. Similarly, the percentage of abnormal morphology, head size and shapes such as micro heads, macro heads, tapered, thin, round, pyri and amorphous forms was significantly more in teratozoospermic and oligoasthenoteratozoospermic groups compared with normal group ($P<0.05$), however no significant difference was observed between asthenozoospermic and normal group. The percentage of spermatozoa with normal acrosome was also significantly more in normal group compared with teratozoospermic and oligoasthenoteratozoospermic groups ($P<0.05$), whereas no significant difference was observed between asthenozoospermic and normal group.

Sperm kinematic parameters between normal and abnormal groups were presented in Table 3. The average sperm count was significantly more in normal group compared with all abnormal groups ($P<0.05$). In motility parameters, type a (progressive) motility was significantly more in normal group compared with asthenozoospermic, oligoasthenoteratozoospermic groups ($P<0.05$), however no significant difference was seen between normal and teratozoospermic group. Type b (sluggish) motility was significantly more in normal group compared with all abnormal groups ($P<0.05$), Type c (non-progressive) motility was significantly more in asthenozoospermic group compared with normal group ($P<0.05$), where as type d (immotile) sperms were significantly more in all abnormal groups compared with normal group ($P<0.05$). The percentage of sperm progressive motility (a+b) was significantly more in normal group compared with all abnormal groups ($P<0.05$). A significant difference in sperm kinetic parameters such as VCL, VSL and VAP was observed between normal and abnormal groups ($P<0.05$). Similarly, a significant difference in ALH was observed in asthenozoospermic and oligoasthenoteratozoospermic groups compared with normal

Table 3 Comparison of sperm motility and kinetic parameters between normal and abnormal groups

Parameters	Normal group	Abnormal group		
	N	T	A	OAT
Count (millions/ml)	144.7±72.80	108.4±70.44*	100.0±55.67*	6.91±4.15*
Type a (%) (progressive)	25.50±10.25	24.93±10.27	7.22±3.94*	9.75±8.45*
Type b (%) (sluggish)	39.11±12.50	32.74±12.33*	15.61±5.99*	13.78±8.83*
Type c (%) (non- progressive)	21.33±6.85	22.14±6.60	25.41±8.33*	15.40±8.41
Type d (%) (immotile)	13.76 ±11.12	20.01±12.70*	51.74±15.39*	61.01±16.68*
a+b (%)	64.61±13.68	57.67±13.45*	22.83±8.01*	23.53±13.95*
VCL (μms^{-1})	53.79±10.47	51.04±10.87*	35.15±7.20*	41.82±14.69*
VSL (μms^{-1})	25.88±6.35	22.83±6.86*	15.56±4.61*	21.12±9.99*
VAP (μms^{-1})	37.41±6.85	35.09±7.12*	23.66±5.21*	28.46±10.33*
ALH (μm)	2.15±0.69	2.05±0.60	1.54±0.60*	1.68±0.83*

values are expressed as±SD

VCL curvelinear velocity, *VSL* straight line velocity, *VAP* average path velocity, *ALH* amplitude of lateral head displacement, *LIN* linearity, *STR* straightness, *WOB* wobble, *ALH* amplitude of lateral head displacement

* $P<0.05$ is considered as statistically significant when compared to normozoospermic group

group ($P<0.05$), however, no significant difference in ALH was observed between normal and teratozoospermic group.

Table 4 shows the mean percentage of sperm nuclear DNA fragmentation index (DFI) as revealed by SCD which was significantly higher in all abnormal groups compared with normal group ($P<0.05$). Significantly higher percentage of CMA3 positive spermatozoa was observed in teratozoospermic and oligoasthenoteratozoospermic groups compared with normal group ($P<0.05$), where as no significant difference was seen in asthenozoospermic group compared with normal group. Similarly, presence of histones in spermatozoa as revealed by AB staining was significantly higher in teratozoospermic and oligoasthenoteratozoospermic groups compared with normal group ($P<0.05$), however no significant difference was seen in asthenozoospermic group compared with normal group.

Discussion

Spermatogenesis is a complex process of all events involving in maturation of primary spermatogonia into a fully mature spermatozoa occurring in the testes. Further, remodeling of sperm shaping takes place during the epididymal passage to produce a homogenous population of spermatozoa with all necessary specific morphological and functional features of sperm for attaining maximum probability of fertilization of an oocyte [19, 20]. However, the process of spermatogenesis is not an efficient one, with a large number of abnormal forms being produced [21]. In certain abnormal states, the number of these abnormal forms increases further, resulting in an inefficient fertilization process, increased aneuploidy and pregnancy wastage. The types of these abnormalities may comprise of morphological variations in shape, altered motility, and defective integrity of DNA or chromatin packing abnormalities.

Assessment of fertility potential of gametes is an important factor to be identified before performing any assisted reproductive technique to ensure predictive outcome. Poor fertilization rates might be related to failed oocyte activation process or alternatively due to sperm related factors [22]. Traditionally, semen parameters like sperm count, motility and morphology were considered for assessment of male factor. Of these, sperm morphology is much emphasized recently and is proven to be one of the important predictors in determining the fertilizing capacity of sperm both in vivo and in vitro conditions [23–25]. Several clinical studies have demonstrated a clear association between sperm head morphology and improvement in IVF success rate [4, 26, 27]. However, other works showed lack of correlation between these parameters [28, 29]. The conventional methods for the evaluation of sperm morphology are highly variable causing difficulty in interpretation of results although several efforts have been made to overcome these problems. Recently developed techniques such as CASA, enable us to analyze sperm morphology in better detail and makes detection of subtle variations in sperm head morphometry possible. Another new test for evaluation of male infertility is DNA fragmentation analysis, which has proven to be reliable for assessment of sperm genomic integrity. It is now widely accepted and has gained clinical importance as the patients with infertility are reported to have high DNA fragmentation rate in comparison with fertile patients [30–32]. Furthermore, DNA fragmentation test is now considered to have a better diagnostic and predictive capability than conventional semen parameters and has become an important diagnostic tool for the assessment of semen quality in ART laboratories [8, 9].

Several studies have demonstrated a positive correlation between abnormal sperm morphology and DNA fragmentation rate [8, 33, 34]. The most common sperm morphological abnormalities reported were tapered, thin, pyri, amorphous heads and acrosome related abnormalities. Tapered heads are usually associated with overall increase in head length with minor deviation in width and the percentage coverage of acrosome in head region is decreased. As a result, chromatin might be poorly packed in the last step of chromatin condensation process with an increase incidence of chromosomal aneuploidy. This explanation is supported by several studies demonstrating that spermatozoa with

Table 4 Comparison of DNA fragmentation index and chromatin packing quality between normal and abnormal groups

Parameters	Normal group	Abnormal group		
	N	T	A	OAT
DFI (%)	18.27±7.19	27.56±9.96*	36.06±11.56*	38.15±13.91*
CMA3 (%)	32.50±7.26	42.88±13.24*	31.60±4.95	52.77±10.68*
AB (%)	26.25±8.55	35.40±11.89*	27.30±6.45	46.63±9.59*

values are expressed as±SD

DFI DNA fragmentation index, *CMA3* chromomycin A3, *AB* aniline blue

* $P<0.05$ is considered as statistically significant when compared to normozoospermic group

tapered or elongated head contain immature chromatin as revealed by AB staining method [35, 36]. Spermatozoa with tapered heads were also reported to have a higher incidence of failed fertilization rates post ICSI due to probable chromatin abnormalities [22]. In addition, spermatozoa with abnormal morphology were also reported to have compromised motility parameters such as rapid and progressive movement. These observations were more specific for spermatozoa with tapered or pyriform heads [21]. Macro head is another morphological abnormality reported to have a very high rate of chromatin condensation abnormalities which might be due to spermatozoa originating from germ cells with impaired meiotic divisions [37]. Similarly, the incidence of chromosomal errors in human spermatozoa with deformed heads such as amorphous form, was reported to be four times higher than sperm with normal morphology [38]. This is further supported by Abdelrazik et al. [39] who analyzed sperm morphology using computer assisted morphometry and demonstrated that spermatozoa with abnormal forms in particular amorphous and micro heads have higher DNA fragmentation rate compared with other forms of head abnormalities. Later studies by Sheikh et al. also confirmed that DNA fragmentation rate was higher in sperms with abnormal morphology [32]. Further studies by Tang et al. reported specific abnormal forms such as tapered and amorphous forms had higher rates of chromosomal abnormalities and DNA fragmentation [40]. Recently, Daris et al. further proved that specific head abnormalities, especially amorphous heads had elevated degree of DNA fragmentation [41]. These abnormal forms containing immature chromatin and high rates of DNA fragmentation results in increased incidence of aneuploidy and mutations in germ line [35, 42]. In the present study too the DFI was significantly higher in morphological abnormal groups compared with normal group. These findings are in agreement with previous studies conducted on infertile patients by several authors [32, 39–41].

Abnormal head sperm morphology was also shown to have a negative correlation with sperm motility parameters [21, 43, 44]. One of the possible physiological explanations for altered motility might be due to compromised hydrodynamic efficiency of sperm resulting from abnormal head shape. Further, sperm with abnormal head shapes may have inherent defects in intracellular events which might be involved in energy production and transduction events within the cell. This explanation is supported by several studies that morphologically abnormal sperms are less viable and either are immotile or swim significantly slower than morphologically normal sperms [21, 43, 44]. Further, the velocity parameters such as VSL and VCL are also reported to be significantly affected by sperm morphology [45]. This is further supported by Aydos et al. showing a negative correlation between abnormal sperm morphology and motility parameters [46]. In the present study, the sperm count, type a and type b motility were significantly higher in normal group compared with abnormal group, where as type d motility was more in abnormal groups compared with normal group which was significant. These findings are corroborated with earlier studies, analyzing the relationship between abnormal sperm morphology and motility parameters [21, 43–46].

Sperm maturation process involves the replacement of somatic cell histones with basic transition proteins and final restoration with sperm specific protamines resulting in highly compacted chromatin with hydrodynamic nucleus and the specific morphological form of the spermatozoa [47, 48]. Any deviations in this maturation process of replacement of histones by protamines may result in poor chromatin packing and severe morphologically abnormal sperms, having a significant influence on fertilization rate [24, 49]. Similarly, several studies have confirmed that poor chromatin packing may lead to sperm decondensation failures post ICSI which affects the fertilization process [50–52]. Furthermore, several studies have also demonstrated a strong correlation between abnormal protamination and sperm DNA fragmentation [53–55]. Protamine confers a high level compactness to sperm nucleus during spermatogenesis and abnormal protamination may result in increased susceptibility of the paternal genome to physical and chemical damage leading to DNA fragmentation. In the present study a significant positive correlation was observed between CMA3 positive and abnormal groups when compared with normal group. These findings are consistent with earlier works done by several authors [53–56].

In conclusion, the results of the present study demonstrated that infertile patients with male factor have high percentage of morphologically abnormal forms. Spermatozoa with abnormal head size and shapes such as micro, macro, tapered, thin, round, pyri and amorphous forms and compromised motility parameters showed high degree of DNA fragmentation and high levels of CMA3 positive. Fragmentation of DNA in human spermatozoa is associated with decreased fertilization and poor pregnancy outcome. This finding has clinical relevance where sample with increased number of these abnormalities has a bad prognosis and avoiding the use of these forms during sperm selection in ICSI may improve the results. Further, a multi-diagnostic approach using CASA for morphology, DNA fragmentation analysis for DNA integrity and CMA3 staining for protamine status can be applied clinically as a part of semen quality evaluation prior to ART procedures for a better prognostic evaluation.

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