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Sperm chromatin packaging as an indicator of in-vitro fertilization rates

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The development of a sequential diagnostic schedule for patients consulting for infertility disturbances would be an ideal method of approach for clinicians in the absence of an aetiological or pathophysiological diagnosis. Since sperm morphology recorded by strict criteria has often been correlated with fertilization failure, the present study aimed to evaluate the relationship between normal morphology as well as in-vitro fertilization (IVF) rates, with chromatin staining among fertile and subfertile men. Two semen smears were prepared from each specimen obtained from 72 men to record normal morphology and chromatin packaging as recorded by chromomycin A_3 (CMA_3) staining. Following the semen analyses, the 72 men were divided into the two morphological groups, namely <4% and >4% normal forms. Significantly different percentages of CMA_3 staining (mean ± SE) were recorded between the two morphological groups, namely 65.9% ± 3.5 and 44.5% ± 1.7 (P ± 0.001). A highly negative significant correlation existed between percentage of normal morphology as recorded by strict criteria and CMA_3 staining. A highly significant and positive correlation was recorded for normal morphology and IVF rates (r ± 0.45, P ± 0.0001). A significant negative correlation (r ± –0.51, P ± 0.0001) existed between CMA_3 values and IVF rates. The discriminating power of nuclear maturity, as recorded by CMA_3 staining, to identify abnormal morphology values and poor IVF rates was calculated with receiver operator characteristic (ROC) analyses. The areas under the ROC curves were 0.86 for sperm morphology and 0.74 for IVF rates. The calculated threshold values for CMA_3 staining to distinguish between morphology groups were 48 and 50% for IVF. Chromatin packaging assessment is a valuable addition to the sequential diagnostic programme in an assisted reproductive arena.

Key words: IVF/morphology/sperm chromatin

Introduction

Semen quality is traditionally determined according to sperm concentration, motility and morphological features in a given ejaculate (World Health Organization, 1987). Likewise, it is accepted that an association exists between sperm morphology as recorded by strict criteria and in-vitro fertilization (IVF) rates (Kruger *et al*., 1986; Liu and Baker, 1988; Oehninger *et al*., 1988). Sperm morphology is also related to the incidence of other sperm deficiencies, including poor zona pellucida binding (Franken *et al*., 1996) and penetration (Lanzendorf *et al*., 1994), a poor response to agonists that modulate intracellular calcium concentrations (Oehninger *et al.,* 1994) and a high content of creatine kinase (Huszar *et al*., 1994).

Poor chromatin packaging as indicated by elevated chromomycin A_3 (CMA_3) staining values (>40%) and possible DNA damage, may contribute to failure of sperm decondensation after intracytoplasmic sperm injection (ICSI) and subsequently result in fertilization failure (Sakkas *et al*., 1995, 1996; Bianchi *et al*., 1996a; Lopes *et al*., 1998).

Several techniques have been described such as the sperm chromatin structure assay (SCSA) which uses the metachromatic properties of Acridine Orange; other dye techniques include the use of Aniline Blue (Henkel *et al*., 1994) and fluorochromes, for example, Methyl Green (Godowicz 1977), Giemsa stain (Windt *et al*., 1994), ethidium bromide (Filatov *et al*., 1999), Acridine Orange (Evenson *et al*., 1999), and CMA_3 (Bianchi *et al*., 1996b). Chromatin of mature spermatozoa has been shown to possess a varying binding capacity for many of the mentioned dyes and stains. The binding capacity is believed to reflect anomalies in the packing quality due to the modifications of the nucleoprotein components occurring during spermiogenesis. Essentially, binding capacity, i.e. ability of various dyes or stain to bind to the sperm chromatin, involves replacement of histones by protamines and then further stabilization by the formation of intra-inter-molecular disulphide cross-links between the cysteine residues of the protamine molecule (Balhorn, 1989). One of these fluorochromes, CMA_3, has been found to be a useful tool for the detection of both protamine-deficient loosely packaged chromatin and nicked DNA (Manicardi *et al*., 1995). The strong correlation that has been shown to exist between sensitivity to CMA_3 staining and sensitivity to endogenous in-situ nick translation has been reported by various groups (Manicardi *et al*., 1995). Sperm chromatin integrity as reflected in a ratio of single- to double-stranded DNA, as measured by the sperm chromatin structure assay (SCSA), is predictive of infertility and subfertility in a range of species including humans (Evenson, 1986; Evenson *et al*., 1994; Bizzaro *et al*., 1998).

The present study aimed to evaluate the relationship between sperm morphology and the recorded IVF rates, with chromatin staining, among men with >4% and <4% normal spermatozoa.

Materials and methods

Sperm preparation

Tests were performed in a private andrology institution (Drs du Buisson and partners, Pretoriak South Africa). Semen samples from 72 men were collected on the day of the IVF treatment of their wives. Samples were analysed for sperm concentration, percentage motility and forward progression using World Health Organization guidelines (WHO, 1987). Sperm morphology was evaluated by strict criteria (Kruger *et al*., 1986). Two semen smears were prepared from each specimen to record normal morphology and chromatin packaging as recorded by CMA_3 staining (Bianchi *et al*., 1996). Samples were classified as fertile and sub-fertile according to the percentage normal spermatozoa (Kruger *et al*., 1986). Separation of the motile fraction was accomplished following a double-wash swim-up method using Ham's F10 (Gibco BRL, Life Technologies Ltd, Paisley, UK) supplemented with 10% human fetal cord serum obtained from patients free from hepatitis B surface antigens, cytomegalovirus, human immunodeficiency virus, sexually transmitted diseases; all these patients underwent a Caesarean section at the time of fetal cord serum collection.

CMA_3 staining

Semen smears were fixed in methanol/glacial acetic acid 3:1 at 4°C, for 20 min. Slides were allowed to air dry at room temperature for 20 min. For CMA_3 staining (Sigma Chemicals, St Louis, MO, USA), each slide was treated for 20 min with 100 µl CMA_3 solution. The CMA_3 solution contained of 0.25 mg/ml CMA_3 in McIlvane's buffer (Geigy Scientific Tables 1984), pH 7.0, supplemented with 10 mmol/l $MgCl_2$. Slides were rinsed in buffer and mounted with Dabco (Aldrich Chemicals Co., Steinheim, Germany). The slides were then kept at 4°C overnight after which evaluation of fluorescence was performed the following morning using a Nikon Labophot 2 (CFWN10× IMP., Johannesburg, South Africa) fitted with a triple band filter fluoroscein isothiocyanate (FITC), rhodamine, 4′,6-diamidino-2-phenylindole (DAPI) and an Eplan 100× objective. The lens allowed the use of both phase and fluorescence. A total of 200 spermatozoa were randomly evaluated on each slide. Evaluation of CMA_3 staining was done by distinguishing between bright yellow stained spermatozoa (CMA_3 positive) and dull yellow stained spermatozoa (CMA_3 negative). A clear distinction existed between CMA_3 positive and negative spermatozoa, since CMA_3 positive spermatozoa revealed an intensive bright fluorescent yellow appearance. Prior to the onset of the study, intra- and inter-assay variations were recorded for fertile sperm donors.

Standardization of results

During the preliminary experiments, technician and sample coefficients of variation (CV) were recorded. Intra-assay variation was determined by evaluating 100 cells on 10 different microscopic fields (total 1000 cells) from the same semen specimen. Inter-assay variation was accomplished by evaluating staining for CMA_3 by counting 200 cells on five different specimens from the same sperm donor. Coefficient of variation (Mortimer, 1994) for both intra- and inter assay values were calculated using the following formula:

$$\text{CV}\ (\%) = \frac{\text{SD}}{\text{mean}} \times \frac{100}{1}$$

The CV during the standardization of results were <12% in all instances.

In-vitro fertilization

Ovulation induction was achieved using a combination of chlomiphene citrate (Serophene; Serono, Rome, Italy) 100 mg daily on days 4–8 of the woman's menstrual cycle, as well as human menopausal gonadotrophin (HMG, Pergonal; Serono, Rome, Italy. The dosage of HMG was administered according to follicular response as assessed by transvaginal ultrasound and daily serum oestradiol. The stimulation programme was individualized according to the patient's response during previous cycles. In some cases stimulation was accomplished using a combination of gonadotrophin releasing hormone agonist (GnRHa), buserelin nasal spray (Suprefact; Hoechst AG, Frankfurt, Germany) and HMG; HCG was given (10 000 IU, Pregnyl; Organon, Istanbul, Turkey) when two or more follicles reached or exceeded a mean diameter of 18 mm with serum oetradiol concentrations of 1800 pmol.

Follicular aspiration for oocyte collection was performed 36 h following the administration of HCG. Ham's F-10 supplemented with 10% human fetal cord serum was used for insemination and culture medium. Follicular fluid was aspirated into sterile tubes (Falcon Plastics; Becton Dickenson Labware, Franklin Lakes, USA). The maturational stage of the oocytes was microscopically assessed after which the oocytes were rinsed in small Petri dishes (Falcon Plastics Cat 3001 CA, USA) containing 4.0 ml Ham's F10 supplemented with 10% human fetal cord serum. Incubation of the gametes as well as the culturing of embryos took place at 37°C, 5% CO_2 in a centre well Petri dish (Falcon Plastics). No more than three oocytes were incubated in a single Petri dish. Ham's F10 supplemented with human fetal cord serum was used as culture medium. The human fetal cord serum was filtered, inactivated and tested for the infectious diseases, i.e. human immunodeficiency virus (HIV) and hepatitis.

Oocytes were inseminated with 100 000–500 000 motile spermatozoa per ml in accordance to the percentage morphological normal forms present. Corona and cumulus cells were removed 16–18 h after insemination and fertilization was confirmed when two pronuclei were identified. Culturing proceeded until the embryos reached a 6–8-cell stage. Three to four embryos were transferred on day 3 using a Tom Cat catheter (Sherwood Medical, St Louis, MO, USA). CMA_3 staining was performed on all semen samples obtained from the 72 men on the day of the procedure, stained and read the following day.

Statistical analysis

Comparisons between normal sperm morphology and IVF percentage chromatin staining were done using correlation analysis. The discriminating power of chromatin packaging as a screening test for the identification of normal sperm morphology and IVF was illustrated with receiver operating characteristic (ROC) analysis (Altman and Bland, 1994).

Results

Normal sperm morphology (strict criteria)

Following the semen analyses, the 72 men were divided into the two morphological groups, namely <4% and >4% normal forms. Significantly lower percentages of CMA_3 staining (mean ± SE) were recorded between the two morphology groups, namely 65.9% ± 3.5 and 44.5% ± 1.7 ($P = 0.001$), respectively (Table I).

A highly significant negative correlation existed between percentage normal morphology as recorded by strict criteria and CMA_3 staining (Figure 1, $r = -0.59$, $P = 0.0001$). In agreement with previous reports, a significant positive correlation was recorded for normal morphology and IVF rates ($r = 0.45$, $P = 0.0001$). A significant negative correlation (Figure 2, $r = -0.51$, $P = 0.0001$) existed between CMA_3

Table I. Semen characteristics (mean ± SE) and in-vitro fertilization results of 72 patients with chromomycin A_3 staining in sperm morphological groups >4 and <4% normal spermatozoa

% normal forms	% CMA_3 staining	% normal morphology	Sperm concentration ($\times 10^6$/ml)	% motile	No. of oocytes	% oocytes fertilized
<4% (n = 11)	65.9 ± 3.5[a]	1.4 ± 0.2[c]	22.5 ± 7.3[e]	60.0 ± 3.0	6.2 ± 1.4[g]	14.9 ± 5.5[i]
>4% (n = 61)	44.5 ± 1.7[b]	16.2 ± 1.1[d]	62.5 ± 4.0[f]	61.7 ± 1.7	6.9 ± 1.2[h]	57.3 ± 3.7[j]

Mann–Whitney U-test: [a] versus [b]: $P < 0.01$; [c] versus [d]; $P < 0.01$; [e] versus [f]; $P < 0.01$; [g] versus [h]: $P > 0.05$; [i] versus [j]: $P < 0.01$.

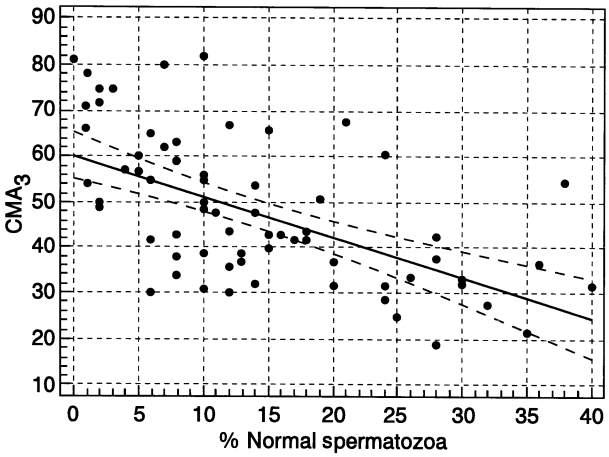

Figure 1. Scatter diagram of chromomycin A_3 (CMA_3) staining and percentage normal sperm morphology of 72 men. The solid line indicates the regression line representing the relationship between CMA_3 staining and the percentage of morphologically normal spermatozoa in raw semen. The dashed lines indicate the 95% confidence interval.

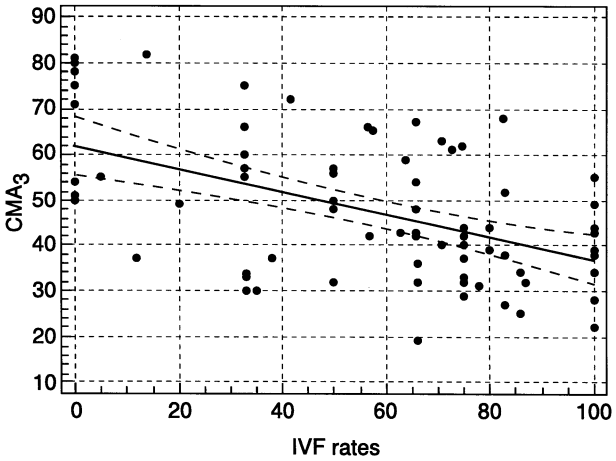

Figure 2. Scatter diagram of chromomycin A_3 (CMA_3) staining in-vitro fertilization (IVF) rates. The solid line indicates the regression line representing the relationship between CMA_3 staining and IVF rates. The dashed lines indicate the 95% confidence interval.

values and IVF results. The mean number of oocytes retrieved and the mean fertilization rate among the <4% and >4% morphology groups are shown in Table I. Fertilization rate was defined as the number of oocytes fertilized when the extrusion of the second polar body was observed 18 h after insemination.

The discriminating power of nuclear maturity, as recorded by CMA_3 staining, to identify abnormal morphology values

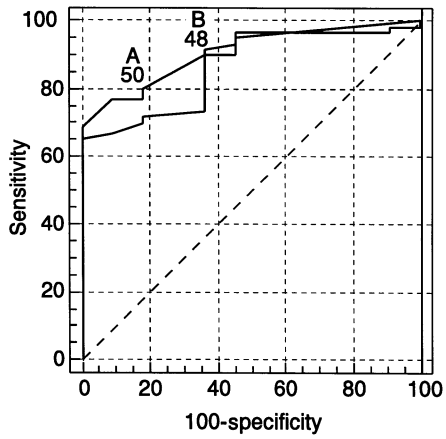

Figure 3. Receiver operating characteristic analyses of chromomycin A_3 (CMA_3) staining, percentage normal sperm morphology and in-vitro fertilization (IVF) rates. Optimal prediction of IVF occurred at CMA_3 values of >50% (**A**), while morphological group prediction occurred at CMA_3 values of >48% (**B**).

and IVF rates with <60% of metaphase II oocytes fertilized, was calculated with ROC analyses. During ROC analyses (Figure 3), varying sperm morphology values were used to calculate the optimum sensitivity and specificity values for CMA_3 staining and IVF rates. The areas under the ROC curves were 0.74 for IVF and 0.86 for CMA_3 staining. The calculated threshold value for CMA_3 staining, to distinguish between the selected morphology groups, was 48%. When IVF rates were used in the ROC analysis, the CMA_3 threshold value for accurate prediction was 50%.

Discussion

The successful implementation of ICSI has provided a unique method of allowing couples suffering from severe male infertility to achieve their reproductive goals. ICSI frequently provides solutions to clinicians often in the absence of an aetiological or pathophysiological diagnosis. We have been promoters of a sequential, multi-step diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics (Oehninger *et al*., 1991).

It is well documented that selection of motile spermatozoa by swim-up can improve the quality of the subfertile spermatozoa recovered, with respect to morphology (Menkveld *et al*., 1991), motility (Ng *et al*., 1992) and nuclear maturity (Le Lannou and Blanchard, 1988). The evaluation of DNA status is indicated in cases where repeated centrifugation of spermatozoa was used to prepare the spermatozoa. A significant increase

in amounts of reactive oxygen was reported, which correlated significantly with DNA damage detected in the spermatozoa (Twigg *et al*., 1998). Several workers in the field of assisted reproduction documented the clinical relevance of strict sperm morphology, i.e. significantly lower fertilization rates reported during IVF treatment (Oehninger *et al*., 1991) as well as impaired sperm–mucus interaction and reduced fertilization capacity under natural conditions of conception (Eggert-Kruse *et al*., 1995).

The power of the morphology of a given sperm sample (>4% and <4% normal forms) to predict the CMA_3 staining quality of the sample was reflected in Figure 3 by the area under the ROC curve, namely 86%, while the area under the curve for IVF was 74% for the selected morphological groups. An area under the curve of 0.86 implies that a randomly selected individual with CMA_3 staining of >48% will have increased CMA_3 staining in 86% of cases. The confidence interval (0.8–0.96) does not include 0.5, implying that CMA_3 staining has the ability to distinguish between morphology groups >4% and <4%. When CMA_3 staining was used as a discriminator of sperm morphology, the sensitivity, i.e. the probability that the percentage CMA_3 staining would be elevated (>48%) in the presence of poor sperm morphology, was calculated at 65%. The specificity, i.e. the probability that CMA_3 staining would reflect decreased staining values (<48%) among the sperm morphology group >4% normal forms, was 100%.

When CMA_3 staining was used as a discriminator of IVF success (>50% oocytes fertilized) a sensitivity of 73% and specificity of 75% was recorded. The 95% CI did not include 0.5 (0.60–0.83), meaning that CMA_3 staining could distinguish between IVF success and failure. An area under the curve of 0.90 means that a randomly selected individual with CMA_3 staining of >50% will fertilize >50% of metaphase II oocytes in 90% of cases.

Although it is evident that spermatozoa in semen containing a high number of abnormal forms has a reduced fertilizing potential, the true anomalies present in abnormal sperm cells have only partially been characterized. Specific biochemical markers have been associated with abnormal spermatozoa, possibly the most important being the association with reactive oxygen species production (Aitken and Clarkson, 1988) and the enhanced creatine phosphokinase activity present in abnormal spermatozoa (Huszar *et al*., 1994). The present data are in close agreement with the conclusions of other studies (Bianchi *et al*., 1996), that poor spermatozoa exhibiting abnormal morphology are more likely to possess loosely packaged chromatin. It appears that abnormal sperm morphology is an overall indicator of spermatozoa that have failed to progress through a complete spermiogenesis. As a consequence they may display characteristics typical of immature spermatozoa, which is also evident in the biochemical composition (Huszar *et al*., 1994).

A normal-shaped sperm head may contain chromosomes with microdeletions, aneuploidy, DNA strand breaks and abnormal sperm chromatin structure, and yet fertilize an oocyte (Evenson *et al*., 1999). Spano *et al*. (1998) used the SCSA and illustrated that the results can be influenced by the age of the sperm donor, smoking habits, the presence of leukocytes and immature germ cells in the ejaculate and the duration of sexual abstinence. During a recent study (Sakkas *et al*., 1996) patients with increased CMA_3 staining (>40%) also had more condensed spermatozoa in unfertilized ICSI oocytes as compared to oocytes from patients with lower CMA_3 staining (<30%). The percentage of spermatozoa which had the capacity to initiate decondensation in unfertilized oocytes was not influenced by morphology or CMA_3 staining (Sakkas *et al*., 1996). Lopes *et al*. (1998) also showed that DNA damage in spermatozoa might contribute to fertilization failure after ICSI. Men undergoing fertility treatment revealed a general association between impaired sperm quality, as recorded by conventional characteristics, and the appearance of spermatozoa with poor chromatin condensation in the ejaculate as expressed by DNA fluorescence intensity (Evenson *et al*., 1999).

Chromatin hypocondensation, depicted by increased fluorescence, was present in different degrees in different sperm samples (Engh *et al*., 1992). It is therefore logical to assume that poor chromatin packing as indicated by an increase percentage of spermatozoa showing intensive CMA_3 staining, may contribute to failure of sperm decondensation after ICSI and subsequently result in fertilization failure. Although it is not postulated that fertilization is due to a single sperm defect, it seems likely that poor chromatin packaging may contribute to a failure in the decondensation process. Auger *et al*. (1990) used acidic Aniline Blue staining to detect chromatin defects of sperm nuclei related to nucleoprotein content as associated with DNA. Semen characteristics that discriminated significantly between fertile and possible infertile men were the percentage of normal spermatozoa, semen volume and the percentage of mature sperm heads. These results indicate that the addition of the evaluation of sperm head maturity to routine semen analysis improves the assessment of fertility in men.

Spermatozoa collected from the epididymis of patients with irreparable obstructive azoospermia fertilized a significantly higher proportion of oocytes during ICSI when spermatozoa were aggressively immobilized. CMA_3 staining could therefore be used as a diagnostic test to identify spermatozoa with elevated staining levels (indicative of poor DNA packaging). Thus patients with a high percentage of stained cells (poor chromatin packaging) could benefit from ICSI provided that the above-mentioned sperm-immobilizing technique is used (Yanagimachi, 1988).

Since ICSI overrides deficiencies in sperm motility, zona and oolemma binding and leaves the onus of successful completion of fertilization upon the sperm nucleus, it is suggested that chromatin packaging assessments should be included as a complementary assay to the sequential diagnostic approach of the male factor patients.

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