CLINICAL ASSISTED REPRODUCTION

Chromatin Packaging as an Indicator of Human Sperm Dysfunction

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Purpose: Understanding the causes of fertilization failure is an important research field in assisted reproductive programs. The present study aimed to evaluate the possible relationship between chromatin packaging quality (CMA₃ staining) and (i) normal morphology and (ii) its ability to predict the functional integrity of spermatozoa in both in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatment programs.

Methods: Semen of 140 men from IVF and ICSI couples were analyzed for sperm concentration, motility, morphology, and chromatin packaging (CMA₃). For CMA₃ classification, two cutoff values were used, namely, $44.5\% \pm 13$ and 1 SD above the mean, i.e., 57.5% (rounded off to 60%). IVF and ICSI data were stratified using three basic cutoff values for CMA₃ staining, namely, <44%, 44–60%, and >60%.

Results: Based on CMA₃ results patients were divided into four groups, namely, group A, <44% CMA₃ (n = 15, IVF); group B, $\geq 44\%$ and <60% CMA₃ (n = 39, IVF); group C, $\geq 60\%$ CMA₃ (n = 45 IVF); and group D, $\geq 60\%$ CMA₃ (n = 41 ICSI). During receiver operator characteristic analyses the estimated cutoff value for CMA₃ staining, to distinguish between <4% and $\geq 4\%$ morphology groups, was 60%. The area under the curve was 0.89, sensitivity of 75%, and specificity of 100%. When IVF rates of >60% and < 60% were used, the optimal CMA₃ value for prediction of fertil-

ization success again was recorded at 60%. The area under the curve was 0.76, sensitivity of 81.5%, and specificity of 63.6%.

Conclusions: Chromatin packaging assessments should be included as a complementary assay to the sequential diagnostic approach of the male-factor patients.

KEY WORDS: Sperm chromatin; morphology; in vitro fertilization; intracytoplasmic sperm injection.

INTRODUCTION

Sperm dysfunction is one of the most common single causes of male infertility, yet remarkably our knowledge of the cellular and biochemical basis for this condition is very limited. Indeed, our understanding of the physiology of the normal human spermatozoon, let alone the dysfunctional spermatozoon, is elementary. The successful implementation of intracytoplasmic sperm injection (ICSI) has provided a unique means to allow couples diagnosed with severe male infertility to achieve their reproductive goal (1). However, despite the great therapeutic advantages of the techniques, in vitro fertilization (IVF) and ICSI provides solutions to clinicians often in the absence of an etiologic or pathophysiological diagnosis.

The underlying cause for their infertility are generally related to specific sperm defects that generally comprise low counts ($<1 \times 10^6/\text{ml}$) and/or low motility ($<30\%$) and/or severe teratozoospermic men (2). Patients with these semen parameters are usually directly referred to the ICSI program. ICSI recently

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also has been applied to patients with obstructive azoospermia, since ICSI also is successfully applied using epididymal and testicular spermatozoa (3,4).

Current important issues being investigated among couples attending assisted reproductive programs include the determination of chromosomal abnormalities and sperm functional quality (5). The need to employ a multidagnostic approach during the clinical workup has been underlined by the participants of a Consensus Workshop in Advanced Andrology and it suggested that sperm functional assays should become part of the male diagnostic program (6–8).

Sperm chromatin packaging as an indicator for fertilization in assisted reproduction recently has been underlined (9–11). To evaluate the chromatin of mammalian sperm, numerous dyes and fluorochromes have been used, for example, aniline blue, methyl green, giemsa stain, ethidium bromide, acridine orange, and chromomycin A₃ (12,13). The binding capacity of these dyes is believed to reflect anomalies in the chromatin packing quality due the modifications of the nucleoprotein components occurring during spermiogenesis. Basically it involves replacement of histones by protamines and then further stabilization by the formation of intra- and intermolecular disulfide cross-links among the cysteine residues of the protamine molecule (13).

The aim of this study was to evaluate the possible relationship between CMA₃ staining and (i) normal morphology and (ii) its ability to predict the functional integrity of spermatozoa in both IVF and ICSI treatment programs.

MATERIAL AND METHODS

Experimental Design

Based on semen parameters, 140 consecutive patients were directed to either the IVF or ICSI program. Patients with <4% normal forms, $<1 \times 10^6$ sperm/ml, and $\leq 40\%$ motile cells (except for one patient with a motility of 50%) and a history of repeated failed IVF were admitted to the ICSI programme. Patients in the IVF and ICSI program were classified according to CMA₃ staining. The criteria for CMA₃ classification were based on the results obtained during a previous study, where $44.5\% \pm 13$ was reported to be a cutoff value for CMA₃ staining among fertile and subfertile men (14). This value was similar to that reported by others (11,12). In order

to analyze the data of the present study; we calculated a second cutoff value estimated at 60.0% recorded as 1 SD above 44.5%. Accordingly, the data were stratified using three basic cutoff values for CMA₃ staining, namely <44%, >44–60%, and >60%. Data were analyzed according to CMA₃ staining values of 44.5% and 60% as cutoff values. Each CMA₃ category of patients were then further divided into two subgroups, namely <4% normal forms and $\geq 4\%$ normal forms.

Ovulation Induction

Ovulation induction protocols were adapted to individual needs. A “long” and “short” protocol was the most commonly used. In the long protocol, Buserelin (Suprefact, Hoechst, IHD, Johannesburg, South Africa), Lucrin [gonadotropin-releasing hormone agonist (GnRHa), Abbott, IHD, Johannesburg, SA], and Gonal-F [recombinant follicle-stimulating hormone (FSH), Serono, Pty (Ltd), Johannesburg, SA] were used. The long protocol treatment was induced in the midluteal or late luteal phase and the short protocol administration of GnRHa begins in the early follicular phase. Both treatments continued until the day of human chorionic gonadotropin (hCG) (Profasi, Serono, Pty (Ltd), Johannesburg, SA) administration. FSH (Metrodin, Serono, Pty (Ltd), Johannesburg, SA) treatment was started after desensitization was achieved. The dose of FSH was tailored to the patient's response. When the largest follicle reached a diameter of 18 mm and the estradiol serum levels indicated a satisfactory follicular response, 10,000 IU hCG was administered.

In Vitro Fertilization

Metaphase II oocytes and embryos were cultured as suggested in the IVF Science and Medi-Cult literature (15). Shortly, culture dishes (Falcon 1006, Becton Dickinson GmbH, Heidelberg) were prepared using 24-hr preequilibrated culture media (IVF-50; Scandinavian IVF Science products, Gothenburg, Sweden; Universal IVF medium; Medi-Cult, MØllehaven, Jyllinge) and Sigma mineral oil (Sigma-M-8410, embryo tested, Sigma-Aldrich, Johannesburg, SA). Three milliliters IVF-50 or Universal IVF medium was incubated in Falcon 2058 tubes (Becton Dickinson GmbH, Heidelberg) for the final swim-up or suspension of spermatozoa after employing a density gradient column separation. Spermatozoa were washed in ASP-100 (Scandinavian IVF Science prod-

ucts, Gothenburg, Sweden) or flushing medium (Medi-Cult, Møllehaven, Jyllinge) and layered with 1.5 ml IVF-50 or Universal IVF. Insemination and oocyte culturing took place in IVF-50 or Universal IVF medium. On day 1, oocytes were denuded and fertilization assessed. Zygotes were transferred into six 50- μ l droplets IVF-50 (Scandinavian IVF Science products, Gothenburg, Sweden) or Universal IVF (Medi-Cult, Møllehaven, Jyllinge).

Two- to four-cell embryos were assessed on day 2 and transferred to prepared dishes, containing six 50- μ l droplets IVF-50 or Universal IVF medium. Culturing of day 3 embryos (8–10 cells) and embryo transfer took place in G2.2 (Scandinavian IVF Science products, Gothenburg, Sweden) or M3 Medium (Medi-Cult, Møllehaven, Jyllinge).

The quality of the embryos were evaluated according to the following system, namely grades I, II, III, and IV. Grade I embryos were those embryos where the blastomeres were equal in size and with no fragments, whereas grade II embryos contained minor fragmentations, with equal-sized blastomeres. When fragments consisted of more than one third but less than two thirds of the volume of the embryo and/or blastomeres were of different sizes, the embryos were graded as grade III. Grade IV embryos showed a high degree of fragmentation (more than two thirds of the volume of the embryo) and blastomeres unequal in size.

Intracytoplasmic Sperm Injection

After retrieval the oocytes were incubated in 0.1% hyaluronidase for 1 to 2 min. The oocytes were rinsed (5 times) in fresh HEPES buffered medium. Prior to injection the oocytes (only metaphase II) were placed in 10 μ l HEPES buffered droplets under oil using 1006 Falcon Petri dish. A small volume (2–3 μ l) of prepared spermatozoa was deposited in a droplet of polyvinylpyrrolidone (PVP; Cat. No. 10890001. Medi-Cult, Harri Labs, Randburg, SA). Narishige micromanipulators mounted on a Nikon inverted microscope were used to perform the sperm injection. An individual morphologically normal sperm were immobilized and sucked into a Cook injection pipette (KMPIP-1035). The sperm were injected and the injected oocyte was then incubated according to the IVF protocol.

Semen Preparation

A total of 140 semen samples were obtained from men attending our fertility program. After complete

liquefaction at room temperature, a basic semen analysis was performed according to the World Health Organization (2, 16). In short, recordings were made of the semen volume, concentration spermatozoa motility, and forward progression. Two separate slides were prepared to evaluate the percentage normal cells and the quality chromatin packaging.

For morphology evaluation all slides were stained with Papanicolaou stain, coverslipped, and then assessed for percentage normal cells according to strict criteria (2). The quality chromatin packaging was determined using CMA₃ staining (Sigma Chemicals, St. Louis, MO, Cat. 2659) techniques (11, 12). In short, CMA₃ staining thin smears were prepared, air-dried, and fixed in methanol/acetic acid 3:1 at room temperature for 20 min. The slides were air-dried and stained with 60–100 μ l CMA₃ in a dark chamber for 20 min. Slides were then washed in McIlvaine buffer (17) and mounted using Dabco (Aldrich Chemicals Co., Milwaukee, WI, Cat. No. 29,073-4). Two hundred spermatozoa were evaluated under a Nikon fluorescent microscope (CFWN10X IMP, Johannesburg, SA; Filter Fx 465-495).

Statistical Analyses

The primary statistical methods employed in the studies included Spearman's rank order correlation between IVF and ICSI groups and semen parameters, logistic regression of fertilization defined as "good" or "poor" at some cutoff value (i.e., 60%) calculated by using fertilization rates from previous IVF cycles. In addition, predictive statistics, that is, sensitivity, specificity, and positive predictive value and negative predictive value for CMA₃ staining and IVF and ICSI results are reported. Predictive values were determined with a 2 $\times$ 2 contingency table, while the statistical relevance was calculated with Yates corrected chi-square test. The discriminating power of sperm morphology and in vitro fertilization as a screening test for the identification of chromatin packaging was illustrated with receiver operating characteristics (18).

RESULTS

Patients

Based on this CMA₃ classification, patients were divided into four groups, namely; group A, <44%

Table I. Sperm Morphology Results of 140 Patients Using CMA₃ Staining Values (mean $\pm$ SD) as Discriminator Between Different Groups^a

	CMA ₃ categories			
	Group A <44% IVF (n = 15)	Group B $\geq 44\%$ –60% IVF (n = 39)	Group C $\geq 60\%$ IVF (n = 45)	Group D $\geq 60\%$ ICSI (n = 41)
Percent normal forms of group	26.9% $\pm$ 7.5 (22–31%)	12.6% $\pm$ 6.7 (11–15%)	7.7% $\pm$ 7.5 (5–10%)	4.0% $\pm$ 3.1 (3–5%)
Range for CMA ₃ staining (%)	39% $\pm$ 3 (38–41%)	52% $\pm$ 4 (50–53%)	70.9% $\pm$ 9 (68–73%)	80.9% $\pm$ 9 (77–83%)
Number of cases <4% (% normal forms)	0	0	16 2.1% $\pm$ 1.2 (1.5–2.7%)	23 2.1 $\pm$ 1.1 (1.7–2.7%)
Number of cases $\geq 4\%$ (% normal forms)	15 26.9% $\pm$ 7.5 (22–31%)	39 13.6% $\pm$ 6.7 (11–15%)	29 12.1% $\pm$ 7.4 (9–15%)	18 8.0 $\pm$ 2.1 (7–9%)
Sperm count ($\times 10^6$ /ml)	84.2 $\pm$ 31.0 (66–101 $\times 10^6$ /ml)	60.3 $\pm$ 29.5 (50–69 $\times 10^6$ /ml)	49.5 $\pm$ 33.5 (39–59 $\times 10^6$ /ml)	17.0 $\pm$ 20.2 (10–23 $\times 10^6$ /ml)
Percent motile cells	65.3% $\pm$ 17.2 (55–73%)	57.9% $\pm$ 12.1 (53–61%)	65.6 $\pm$ 10 (61–69%)	37.9 $\pm$ 22 (29–46%)

^a Numbers in parentheses are 95% confidence interval of the mean.

CMA₃ ($n = 15$, IVF); group B, $\geq 44\%$ and $< 60\%$ CMA₃ ($n = 39$ IVF); group C, $\geq 60\%$ CMA₃ ($n = 45$ IVF); and group D, $\geq 60\%$ CMA₃ ($n = 41$ ICSI). Ninety-nine patients were admitted to the IVF program, while 41 received ICSI treatment. Each CMA₃ group's morphological characteristics are represented in Table I.

Sperm Morphology and Fertilization versus CMA₃ Staining

Evaluation of the 140 semen analyses revealed 39 cases diagnosed as normozoospermic ($\geq 14\%$ normal forms), 61 cases as teratozoospermic (4–13% normal forms), and 39 cases as severe teratozoospermic ($< 4\%$ normal forms). As expected, semen parameters were significantly poorer among the ICSI treated patients (group D), compared to that recorded of the IVF group (groups A, B, and C). The percentage of

morphologically normal spermatozoa and the sperm count ($\times 10^6$ /ml) were significantly different in the three IVF and ICSI groups. Percentage motility in the four groups did not differ significantly (Table I). Although the highest fertilization rate (Table II) was found in group A (72.2%), it was not significantly higher than the fertilization rate in group B (64.0%). The fertilization rates reported for group C (43.7%) and group D (35.0%) were significantly lower compared to the fertilization rates in group A (72.2%) and group B (64.0%, $P < 0.0001$).

In the ICSI and IVF groups where CMA₃ staining values of $> 60\%$ were reported the fertilization rates were 35.0% and 43.7%, respectively. This difference was not statistically significant. The cleavage rates as well as embryo quality in the four different groups were similar. Likewise, the number of embryos transferred per patient in the four different groups did not differ (Table II). However the implantation

Table II. Comparison Between Fertilization Rates of 1032 Metaphase II Oocytes Retrieved from 140 Patients and Chromatin Packaging Quality as Recorded by CMA₃ Staining^a

Group	CMA ₃ Value	Number of oocytes	Number of oocytes fertilized	Fertilization rates (%)	Cleavage (%)	Number of embryos transferred	Embryo grading (%)			
							Grade I	Grade II	Grade III	Grade IV
AIVF	<44%	126	91	72a	95.0	3.4 $\pm$ 0.7	41	41	12	6
BIVF	$\geq 44\%$ to 60%	268	171	64.0b	91.0	3.2 $\pm$ 0.8	39	42	12	6
CIVF	$\geq 60\%$	281	123	43.7c	94.0	2.8 $\pm$ 0.6	46	39	11	4
DICSI	$\geq 60\%$	357	124	35.0d	86.0	2.6 $\pm$ 0.9	35	40	14	10

^a Paired Fisher's exact test: a vs. b, $P > 0.05$; a vs. c, $P < 0.05$; a vs. d, $P < 0.05$; b vs. c, $P < 0.05$; b vs. d, $P < 0.05$.

and clinical pregnancy rates of the ICSI and IVF groups with a CMA₃ percentage of >60% were significantly lower (Table III). No significant difference were observed in the implantation and clinical pregnancy rates in the IVF groups with a CMA₃ percentage of <44% and ≥44% to 60% (Table III).

For IVF patients we used a fertilization rate cutoff value of 60% and a CMA₃ staining percentage of 44% and calculated the predictive values for CMA₃ as far as fertilization success is concerned. The positive predictive value was 80%, negative predictive value 49%, sensitivity 22%, and specificity of 93%. The low negative predictive value (NPV) (49%) and sensitivity (22%) implies that CMA₃ staining does not have a high discriminate level in predicting fertilization failure. The positive predictive value (PPV) of 80% and specificity of 93% indicates the power of CMA₃ staining to predict fertilization success. Possible explanation for the low sensitivity is possibly due to the large population of patients ($n = 39$) that revealed slightly elevated CMA₃ (>44%–60%) values but succeeded in fertilizing >60% of the oocytes. Furthermore, using a CMA₃ cutoff value of 60% instead of the 44.5%, the 2×2 contingency table revealed a sensitivity of 49%, specificity of 91%, PPV of 80%, and NPV of 60%.

Receiver Operator Characteristics Curve Analyses

The discriminating power of nuclear maturity, as recorded by CMA₃ staining, to identify abnormal morphology values and fertilization failure in the IVF groups were calculated with receiver operator characteristics (ROC) analyses. During ROC analyses we used 4% normal forms as a cutoff value to calculate the optimum sensitivity and specificity levels for CMA₃ staining. The areas under the curve were 0.89,

Fig. 1. Receiver operator characteristics analyses of CMA₃ staining and percentage normal sperm morphology >4% and <4% normal forms. Optimal prediction of correct morphological classification occurred at CMA₃ values of >61%.

sensitivity 75%, and specificity 100%. During these calculations, the indicated cutoff value for CMA₃ staining, to distinguish between <4% and ≥4% morphology groups, was 60% (Fig. 1).

When in vitro fertilization rates of >60% and <60% were used in the ROC analysis, the optimal CMA₃ value for accurate prediction of fertilization success was again recorded at 60%. The area under the curve was 0.76, sensitivity of 81.5%, and specificity of 63.6% (Fig. 2).

Table III. Comparison Between Pregnancy Outcome and Chromatin Packaging Recorded among four Groups of Patients^a

Group	Implantatin rate (%)		Clinical pregnancy per embryo transfer (%)
	Per embryo transfer	Per aspiration	
A IVF CMA ₃ <44%	43a	40e	32i
B IVF 44 < CMA ₃ < 60	35b	33f	28j
C IVF CMA ₃ < 60	29c	21g	18k
D ICSI CMA ₃ > 60	20d	17h	15l

^a Paired Fisher's exact test: a vs. b, $P > 0.05$; a vs. c, $P < 0.05$; a vs. d, $P < 0.05$; b vs. c, $P < 0.05$; b vs. d, $P < 0.05$; e vs. f, $P > 0.05$; e vs. g, $P < 0.05$; e vs. h, $P < 0.05$; f vs. g, $P < 0.05$; f vs. h, $P < 0.05$; i vs. j, $P > 0.05$; i vs. k, $P < 0.05$; i vs. l, $P < 0.05$; j vs. k, $P < 0.05$; j vs. l, $P < 0.05$.

Fig. 2. Receiver operator characteristics analyses of CMA₃ staining and in vitro fertilization rates of >60% and <60%. Optimal prediction of in vitro fertilization occurred at CMA₃ values of >61%.

DISCUSSION

The development of a sequential analytical program in the diagnostic andrology laboratory, apart from sperm functional tests, should include assays that provide information that will assist clinicians on the therapeutic approach of couples attending an assisted reproductive program. During a Consensus Workshop in Advanced Andrology, four categories of tests were proposed as important sperm functional tests: (i) computer-assisted evaluation of sperm motion characteristics (CASA), (ii) inducibility of the acrosome reaction and bioassays that sequentially assess gamete interaction, including (iii) sperm–zona pellucida binding tests and (iv) sperm–hamster egg penetration assay (8).

The ability of sperm to fertilize is not only closely correlated with its morphology (2) but also to the quality of the chromatin packaging (10–12). The constant remodeling of sperm chromatin that occurs during spermiogenesis and fertilization leads to special interest in the state of the DNA during the process. The changing structural state of sperm chromatin, as revealed by nucleases, and related alterations in methylation levels therefore are of particular interest during condensation and decondensation. These changes in chromatin are of particular interest because the modifications have been demonstrated to

be heritable through successive DNA replication cycles. These changes in chromatin are of particular interest because the modifications have been demonstrated to be heritable through successive DNA replication cycles (19).

During fertilization, the decondensation process is characterized by the degradation of protamines, synthesis of histones, and binding of the histones to the DNA, leading to a restoration of the paternal genome and its transcriptionally active conformation. If an abnormal sperm containing underprotaminated chromatin and nicks in the DNA fertilizes an oocyte, then the fertilization process and ensuing development may not necessarily proceed normally. Investigation of chromatin state and methylation levels, especially during pronuclei formation, therefore may assist in the knowledge of the role played by the paternal component during embryogenesis. This is of interest, particularly as the paternal genome is earmarked for selective expression during early development (20).

Present CMA₃ staining data indicate chromatin packaging quality to be a discriminator of in vitro fertilization success (>60% oocytes fertilized, a sensitivity of 81.5%, and specificity of 63.6% was recorded). The 95% confidence interval did not include 0.5 (0.70 to 0.83), meaning that CMA₃ staining could distinguish between IVF success and failure. An area under the curve of 0.76 means that a randomly selected individual with CMA₃ staining of >60% will fertilize >50% of metaphase II oocytes in 76% of the cases.

Furthermore, CMA₃ staining seems to be an excellent discriminator of sperm morphology, with a sensitivity of 75% and specificity of 100%. Again, the 95% confidence interval did not include 0.5 (0.81 to 0.90), meaning that CMA₃ staining could distinguish between >4% and <4% morphology samples. Groups A and B, that is, patients with ≤44% and ≥44%–60% CMA₃, respectively, contained no severe teratozoospermic (<4% normal forms) patients, while 39 severe teratozoospermic men were classified in groups C and D, that is, men with >60% CMA₃ staining. It is important to note that each laboratory should determine its own cutoff value for CMA₃ staining. Results should be interpreted with caution, since the cutoff value of different institutions might be totally different. It is therefore important to stratify the data from a large patient population in order to identify the gray areas of the test.

Previous reports have shown that a decrease in fertility is not only associated with sperm-displaying abnormal morphology but also revealed an increased

sensitivity of the DNA to denaturation (21). These observations have been confirmed (22,23) whereby morphological abnormal sperm showed both a high level of fluorescence to CMA₃ fluorochrome and a high presence of endogenous nicks in the DNA. Even data including normal sperm morphology (>14%) have been correlated with fertility success; a normal-shaped sperm head may contain chromosomes with microdeletions, aneuploidy, DNA strand breaks, and abnormal sperm chromatin structure and yet fertilize an oocyte. On the other hand, since ICSI overrides deficiencies in sperm motility, zona, and oolemma binding and leaves the successful completion of fertilization upon the sperm nucleus, we suggest that chromatin packaging assessments should be included as a complementary assay to the sequential diagnostic approach of the male factor patients (24).

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