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Chromatin packaging and morphology in ejaculated human spermatozoa: evidence of hidden anomalies in normal spermatozoa

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This study aimed to investigate the association between anomalies in sperm chromatin packaging, morphology and fertilization in patients undergoing routine in-vitro fertilization (IVF) or subzonal insemination (SUZI). Sperm chromatin packaging was assessed using chromomycin A_3 (CMA_3), a fluorochrome specific for guanine–cytosine rich sequences of DNA. One hundred to 150 sperm cells were assessed in 55 patients to compare sperm chromatin packaging and morphology to fertilization after IVF or SUZI. When the morphology and CMA_3 fluorescence of individual spermatozoa was assessed, >75% of the macrocephalic sperm fluoresced in all patients. In contrast, a mean of 37% of the spermatozoa with normal morphology fluoresced in IVF patients compared with 58% of the normal spermatozoa in male factor patients treated by SUZI. SUZI patients displaying a high fluorescence (>70%) in their spermatozoa also had a significantly lower fertilization rate. Lower packaging quality in morphologically normal spermatozoa may represent a major limiting factor in the fertilizing ability of male factor patients. This study confirms that a high percentage of CMA_3 positivity is present in certain forms of male factor infertility and that such a test may be used to distinguish separate populations in morphologically normal spermatozoa.

Key words: chromatin/chromomycin A_3/in-vitro fertilization/male infertility/spermatozoa

Introduction

Semen quality is conventionally determined according to the number, motility and morphology of spermatozoa in an ejaculate (World Health Organization, 1989). In turn, it is generally accepted that an association exists between these semen parameters and fertilizing ability (Mahadevan and Trounson, 1984; Kruger *et al.*, 1986; Liu *et al.*, 1988). Sperm morphology, in particular, seems to be the most significantly related parameter to fertilization rates (Pousette *et al.*, 1986; Oehninger *et al.*, 1988). With the advent of in-vitro fertilization (IVF) and related techniques such as subzonal insemination (SUZI) and intracytoplasmic sperm injection (ICSI), it has become increasingly apparent that the number, motility and morphology of spermatozoa are not always indicative of a male's fertility status. Significantly different fertilization rates have been reported for patients with similar semen parameters, suggesting that a more sensitive test is needed to identify the inherent defects which render certain spermatozoa unable to fertilize (Jeyendran *et al.*, 1989; Wolf *et al.*, 1992).

A failure of the conventional semen parameters to predict fertilization indicates that hidden anomalies, lying at the sperm membrane level or at the chromatin level, should also be evaluated. A number of studies have shown that spermatozoa with abnormal nuclear chromatin organization are more frequent in infertile men than in fertile men (Evenson *et al.*, 1980; Monaco and Rasch, 1982; Foresta *et al.*, 1992). In these studies, specific dyes and fluorochromes were used to assess the chromatin of mature mammalian spermatozoa and relate them to fertilization results. The accessibility of specific dyes and fluorochromes to DNA can give clues to the packaging of the chromatin, which occurs during spermiogenesis. However, the targets of these fluorochromes or dyes vary and may not be adequate in identifying specific anomalies in chromatin structure. For example, Monaco and Rasch (1982) compared DNA fluorochromes specific for GC-rich sequences, mithramycin and chromomycin A_3 (CMA_3), and for AT-rich sequences, 2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole and 4–6, diamidino-2-phenylindole. They concluded that, although fluorescence was evident in spermatids and spermatocytes, a decline in staining with GC-specific dyes during maturation probably reflected changes in protein composition and DNA packaging ratios.

In our own studies (Bianchi *et al.*, 1993), we have found that the amount of protamines present in mature spermatozoa represented at least one of the limiting factors controlling the accessibility of CMA_3 to the DNA. In the same study we postulated that the CMA_3 fluorochrome could be used as a tool in routine laboratory analysis for the rapid screening of certain conditions of subfertility and infertility in man, as it seemed to allow an indirect visualization of protamine-deficient, nicked and partially denatured DNA. In this study we investigate the use of CMA_3 in relation to individual sperm morphology. Furthermore, we assess the relationship between CMA_3 fluorescence, in conjunction with morphology, and the

Table I. Distribution of the semen characteristics in treatment cycles of in-vitro fertilization (IVF) and subzonal insemination (SUZI) according to (a) sperm concentration and motility and (b) morphology

	Sperm motility (%)					
	0–20		21–50		>50	
	IVF	SUZI	IVF	SUZI	IVF	SUZI
Sperm concentration ($\times 10^6$/ml)						
0–5	1	5	1	6	0	2
6–20	0	4	0	7	2	0
>20	0	1	6	4	21	1
Morphology (% normal forms)	IVF	SUZI[a]				
0–20	4	18				
21–40	17	8				
>40	10	2				

[a] No assessment in two cycles due to insufficient spermatozoa.

outcome of fertilization for patients being treated by routine IVF and SUZI.

Materials and methods

Patient characteristics and sperm preparation

Semen samples were all taken from 55 patients undergoing treatment for infertility at the Clinic for Infertility and Gynaecological Endocrinology-WHO Collaborating Centre, University Hospital of Geneva, Geneva, Switzerland. Ejaculated human spermatozoa were collected and the greater part prepared for routine IVF or SUZI. The criteria for selection of patients performing either procedure was: for IVF, an insemination droplet of at least 100 000 motile spermatozoa could be prepared, and for SUZI, <100 000 motile spermatozoa after preparation or patients having failed to achieve fertilization in at least two previous IVF attempts. Table I shows the semen characteristics for the 55 patients (61 cycles) making up the IVF and SUZI groups. Morphology was assessed according to World Health Organization criteria (1989) and progressive motility by visual appraisal using a Makler chamber.

IVF and SUZI were performed using the stimulation protocol and procedures previously published (Urner *et al.*, 1993; Sakkas *et al.*, 1994). Spermatozoa were prepared for both techniques by treating the sample with 3 mM pentoxifylline (Sigma Pharmaceuticals, Buchs, Switzerland) followed by selection using mini-Percoll gradient centrifugation (Ord *et al.*, 1990). From the remains of the semen sample, one slide was prepared for assessment of sperm morphology while the rest was washed in Dulbecco's Ca^{2+}-Mg^{2+} free phosphate-buffered saline (PBS) and centrifuged at 170 *g* for 10 min. The procedure was repeated twice and the washed spermatozoa were fixed in methanol/glacial acetic acid 3:1, at 4°C, for 5 min and then spread on slides.

Chromomycin A_3 staining and assessment of sperm morphology

For CMA_3 (Sigma) staining, each slide was treated for 20 min with 100 µl of CMA_3 solution (0.25 mg/ml in McIlvane buffer, pH 7.0, containing 10 mM $MgCl_2$). Slides were then rinsed in buffer and mounted with buffered glycerol. Fluorescence was performed using a Zeiss Photomikroskop III using a combination of exciter: dichroic: barrier filters of BP 436/10: FT 580: LP 470 and an A-Plan X20 objective lens. This lens allowed the use of both phase and fluorescence. For each patient, a total of 100–150 spermatozoa were randomly observed on duplicate slides. For each single spermatozoon, head morphology (divided in three categories: normal, macrocephalic or amorphous) and fluorescence were assessed.

Table II. The mean percentage of spermatozoa positive to the CMA_3 fluorochrome stain according to sperm concentration and motility

	No. of cycles	% CMA_3 fluorescence[a]
Sperm concentration ($\times 10^6$/ml)		
0–5	14	68.0 ± 18.1
6–20	13	59.8 ± 23.0
>20	34	47.7 ± 16.5[b]
Motility (%)		
0–20	11	71.5 ± 20.6
21–40	22	55.4 ± 21.8
>40	28	48.0 ± 15.0[c]

[a] Values are means ± SD.
[b] $P < 0.05$ compared to 0–5×10^6/ml sperm concentration group.
[c] $P < 0.05$ compared to 0–20% motility group.

Statistical analysis

The means in the different groups were transformed using an arcsin square root transformation and compared for statistical significance using one-way analysis of variance and Scheffé's *F*-test. A test of the correlation between the percentage of spermatozoa positive to the CMA_3 fluorochrome and head morphology per patient was performed using regression analysis.

Results

Chromomycin A_3 positivity in relation to sperm concentration, motility and morphology

Sperm concentration and motility

The mean percentage of spermatozoa fluorescent after CMA_3 treatment declined in relation to increased sperm concentration and motility (Table II). Patients with severe oligozoospermia or asthenozoospermia had a mean (±SD) of 68.0 ± 18.0% and 71.5 ± 20.6% of their spermatozoa fluorescing respectively. A distinct decline in spermatozoa quality was therefore associated with CMA_3 fluorescence.

Morphology

Although a decline in sperm number and motility was associated with increased CMA_3 fluorescence, a comparison of CMA_3 positivity and morphology in individual spermatozoa showed that apparently normal spermatozoa may possess hidden anomalies. By observing fluorescence and morphology in single sperm heads from the two groups of patients, we found that in all samples >75% of the spermatozoa displaying macrocephaly fluoresced brightly while ~50% of the spermatozoa classed as having an amorphous head shape fluoresced (Figure 1). The main difference between the treatment groups was seen in spermatozoa classed as having a normal shape. In patients undergoing routine IVF, a mean (±SD) of 38.2 ± 19.2% of the morphologically normal spermatozoa fluoresced while in the male factor patients treated by SUZI 57.8 ± 27.4% fluoresced; this was significant ($P < 0.05$). In nine

out of 30 samples from male factor patients, >80% of all spermatozoa were fluorescent compared with only one of the IVF samples.

There was no correlation between the percentage of spermatozoa with abnormal head morphology and the percentage of spermatozoa showing fluorescence after CMA_3 staining (Figure 2). Therefore, although macrocephalic spermatozoa were nearly always fluorescent, spermatozoa with amorphous and normal heads vary in chromatin packaging. Samples prepared from the same ejaculate and evaluated independently gave r values of 0.36 and 0.41 when comparing percentage of cells with abnormal head morphology and showing CMA_3 fluorescence for the IVF and SUZI patients respectively (Figure 2).

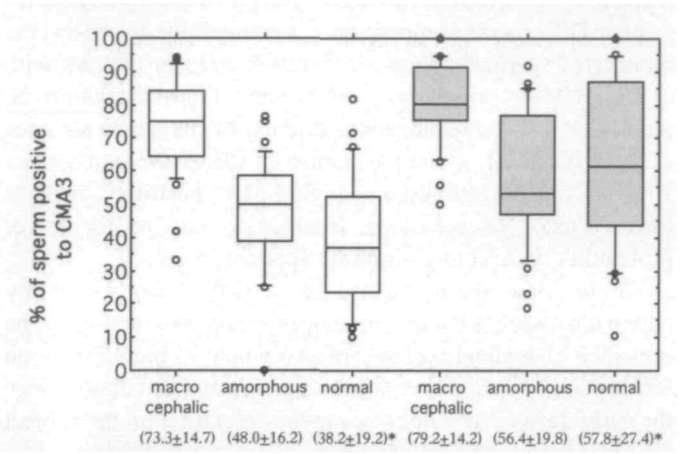

Figure 1. A box plot comparing the different types of sperm morphology to the percentage of spermatozoa positive to the chromomycin A_3 (CMA_3) fluorochrome. The lower 25th and upper 75th percentiles are shown. Two groups are represented, normozoospermic patients (n = 31) treated by routine in-vitro fertilization (IVF, open) and male factor patients (n = 30) treated by subzonal insemination (SUZI, shaded). Individual spermatozoa were assessed for both morphology and CMA_3 fluorescence. The mean (± SD) percentage of spermatozoa positive for CMA_3 fluorescence in each group is given in parentheses. *Percentage of spermatozoa with normal morphology and positive for CMA_3 fluorescence was significantly different ($P < 0.05$) in samples from the IVF and SUZI groups of patients.

Chromomycin A_3 positivity in relation to fertilization, cleavage rates and pregnancy

In-vitro fertilization

There was no association between CMA_3 fluorescence in all spermatozoa and fertilizing ability for patients being treated by routine IVF (data not shown). In seven cases where patients had <20% fertilization rates there was no difference in positivity of sperm to the CMA_3 fluorochrome when compared to cases with fertilization rates >20%. As in routine IVF there is a greater competition between spermatozoa, it could be expected that a motile morphologically normal spermatozoon would fertilize the oocyte. However, no relationship was found between fertilizing ability and CMA_3 fluorescence in morphologically normal spermatozoa in patients treated by routine IVF.

Subzonal insemination

In contrast to the lack of an association in IVF patients, male factor patients treated by SUZI had a significantly lower mean fertilization rate when their spermatozoa showed a high positivity to the CMA_3 fluorochrome. Ten SUZI patients had >70% of spermatozoa positive to the CMA_3 fluorochrome and had a significantly lower ($P < 0.02$) fertilization rate; CMA_3 of >70%: 24.9 ± 15.9 compared with CMA_3 of <70%: 55.7 ± 26.3. The same 10 patients also had >70% fluorescence in their morphologically normal spermatozoa, which was indicative of poor fertilizing ability when using SUZI (Table III).

Embryo cleavage and pregnancies

Once fertilization had occurred, there was no relationship between the ability of a fertilized oocyte to cleave and positivity

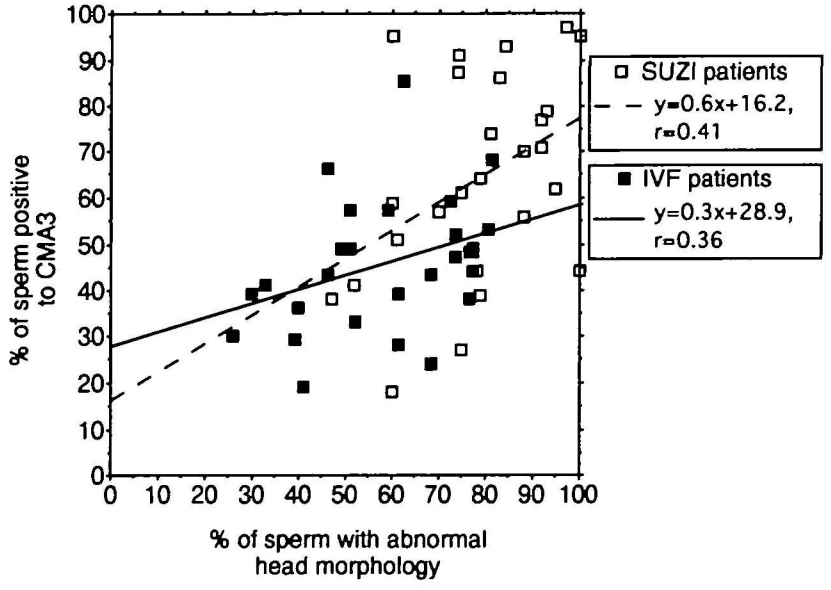

Figure 2. Correlation between the percentages of spermatozoa with abnormal head morphology and spermatozoa positive to the chromomycin A_3 (CMA_3) fluorochrome. Regression equations are given for the normozoospermic patients (n = 31) treated by routine in-vitro fertilization (IVF) and male factor patients (n = 30) treated by subzonal insemination (SUZI).

Table III. Mean fertilization rate of sperm samples from men treated by subzonal insemination (SUZI) in relation to positivity of morphologically normal spermatozoa to the CMA_3 fluorochrome

Percentage of morphologically normal sperm fluorescing	No. of cases[a]	Mean fertilization rate[b]
0–50	10	56.4 ± 25.2
50–70	7	53.6 ± 30.2
> 70	10	24.9 ± 15.9[c]

[a]Only cases which had more than four oocytes treated were included.
[b]Values are means ± SD.
[c]$P < 0.02$ compared to the 0–50 and 50–70 groups.

to the CMA_3 fluorochrome in either treatment group. Five of the 31 IVF cycles resulted in ongoing pregnancies; of the five pregnancies, four of the male partners showed CMA_3 fluorescence values of 20–40%, while one male had 86% of his spermatozoa fluorescing after CMA_3 staining. In the two ongoing pregnancies from the 30 SUZI cycles, the CMA_3 fluorescence was 62 and 77%. Although the numbers are limited, the results suggest that CMA_3 positivity is not related to embryo viability.

Discussion

One of the central events of spermiogenesis is the substitution of the chromatin structural proteins by protamines, allowing a different structural organization to take place in the sperm nucleus (Courtens and Loir, 1981; Balhorn, 1989; Ward and Coffey, 1991). Indeed, as reviewed by Ward and Coffey (1991), chromatin packaging in spermatozoa is very different from that of somatic cells; for example, the mouse sperm cell nucleus is 40-fold smaller than the nucleus of the mouse somatic cell. Due to the tight packaging afforded by the protamines, any modification or absence of these proteins could lead to an anomaly in the packaging process of sperm nuclei and influence sperm quality and fertilizing capacity.

The different models proposed for the binding of protamines to DNA in spermatozoa (Balhorn, 1982; Subirana, 1991) postulate that the polyarginine segment of protamine binds in the minor groove of DNA, cross-linking and neutralizing the phosphodiester backbone. The CMA_3 fluorochrome is also believed to access DNA in the minor groove of the DNA helix (Behr *et al.*, 1969; Berman *et al.*, 1985), a positional preference that can make it a competitor to protamines. CMA_3 has previously been shown to be a useful tool to indicate protamine-depleted spermatozoa (Bianchi *et al.*, 1993). In contrast to the homogeneity of mature mouse spermatozoa, ejaculated human spermatozoa samples were found to exhibit a wide variation both in their responsiveness to the CMA_3 fluorochrome and in the presence of endogenous nicks in the DNA (Bianchi *et al.*, 1993; Sakkas *et al.*, 1995).

Treatment of human sperm samples with protamines eliminates CMA_3 positivity and prevents the identification of endogenous DNA nicks by in-situ nick translation, a technique that reveals damaged DNA (Bianchi *et al.*, 1993; Manicardi *et al.*, 1995). We have now investigated the relationship between CMA_3 positivity and fertilizing capacity in spermatozoa from two populations of patients undergoing IVF and SUZI treatment and found interesting differences in respect to their response to the CMA_3 fluorochrome. In this study we show that the CMA_3 fluorescence provides evidence of: (i) a population of spermatozoa that exhibit normal head morphology but possess loosely packaged chromatin; and (ii) a positive correlation between fertilization failure and CMA_3 fluorescence in morphologically normal spermatozoa in the SUZI treatment, and not in the routine IVF group. Such a difference is most likely related to the different methods of achieving fertilization. In routine IVF, sperm competition is maximal due to the excess number of spermatozoa used; therefore even in patients with a high CMA_3 positivity enough normal sperm should be present to achieve fertilization. In contrast, in semen samples selected for SUZI, where the number of spermatozoa in contact with the oocyte is limited, a high CMA_3 positivity yields a lower chance of achieving fertilization due to the lower probability of selecting a normal spermatozoon.

To this day, sperm quality has mainly been judged by parameters such as number, motility, and morphology. The influence of seminal parameters as a whole or individually on fertility has been a matter of investigation and discussion since the early days of infertility treatment. Literature on the subject is contradictory and results can vary extensively according to methodology of analysis, dyes utilised, interpretation criteria of morphology and technology available. Nonetheless, Bestoffe *et al.* (1982), showed an inverse correlation between percentage of abnormal sperm forms and spontaneous pregnancies in a retrospective study in subfertile couples. This debate has gained new vitality since the new assisted reproduction technologies provided a much more precise tool for evaluation of fertilization rates. Although a proper consensus has not yet been established, it has become widely accepted that normal parameters, defined according to WHO criteria (1989) are associated with high percentages of fertilization and cleavage rates in IVF. Furthermore, many authors have concentrated their attention on the predictive value of morphology (Kruger *et al.*, 1986; Pousette *et al.*, 1986), and it is felt that normal morphology is indicative of normal function, while high levels of morphologically abnormal spermatozoa in the ejaculate are associated with a reduced fertilizing potential (Oehninger *et al.*, 1988). When we compared the morphology and CMA_3 fluorescence in individual spermatozoa, we found a high frequency of CMA_3 positive nuclei among macrocephalic spermatozoa in both treatment groups. This confirms data known on other fluorescent dyes, in particular Acridine Orange, where access to the DNA was achieved only or mostly in cases of decondensed or damaged chromatin (Evenson *et al.*, 1980; 1991; Foresta *et al.*, 1992; Molina *et al.*, 1995; Sailer *et al.*, 1995). Our observations also confirm that macrocephaly is associated with decondensation. Moreover, this is further evidence of the protamine-depleted status of macrocephalic spermatozoa, as CMA_3 is unable to access DNA in the presence of protamines and normally formed disulphide bonds (Bianchi *et al.*, 1993; Kvist, 1982). The fact that fluorescence was still present in the 'amorphous' category but was reduced to 50% probably reflects the fact that this group contained several, and probably

very different head defects, not all of which correspond to a decondensed status of the nuclear chromatin.

In our opinion the most interesting result was the statistically significant difference in fluorescence of normal spermatozoa between the two groups examined, casting a shadow on the value of assessing normal morphology, particularly in the cases of oligoasthenoteratozoospermia (OAT). As proposed by Bartoov *et al.* (1994) such patients may require a more complete sperm evaluation, as normal forms observed by light microscopy do not fully express the anatomical capability hidden in the sperm morphology. Engh *et al.* (1992) also found in a cytofluorimetrical evaluation of 100 male patients undergoing investigations because of infertility that patients with severe OAT presented a higher percentage of fluorescent cells in comparison with normozoospermic patients. Our data confirm this *in situ* with a direct comparison, per individual cell, of fluorescence and morphology. Interestingly, one third of the samples of SUZI patients (usually patients with OAT) presented >80% of fluorescent cells, while only one out of 31 of the IVF patients presented the same features. This suggests that the difference does not only rely on a decrease of the classical sperm parameters but on a defect in the packaging of chromatin, which can affect all the spermatozoa produced by testicular germinal tissue.

This highlights the importance of detecting this population of patients prior to treatment by IVF, as a spermatozoon possessing decondensed chromatin may be unable to achieve syngamy, even upon penetration of the oolemma membrane. When we compared the results of fluorescence to fertilizing capacity in the SUZI patients, we found data to reinforce this hypothesis, as the fertilization rate declines to nearly 20% when a very high number of spermatozoa are fluorescent, a situation that could be taken into account when deciding upon treatment. The fact that cleavage rate is apparently not influenced confirms that the crucial moment is at intra-oocyte decondensation and/or disturbances in its occurrence with all the subsequent processes such as protamine elimination and substitution by histones. Although this result has been observed using the SUZI technique, its relevance to the use of intracytoplasmic sperm injection (ICSI) as a treatment of male factor infertility awaits resolution.

The results from this study suggest that CMA_3 could become a useful tool for evaluation of male factor patients prior to infertility treatment. CMA_3 will distinguish decondensed, protamine-depleted spermatozoa. Moreover, CMA_3 is also useful in identifying DNA-damaged sperm cells as shown by the presence of endogenous nicks in many decondensed CMA_3 positive nuclei (Manicardi *et al.*, 1995). This would make it possible to screen for those patients whose spermiogenesis has resulted in a sperm population containing intrinsic anomalies in the nuclei, regardless of sperm morphology.

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