Chromomycin A_3 Staining as a Useful Tool for Evaluation of Male Fertility

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Purpose: *Our purpose was to investigate the association between percentage chromomycin A_3 (CMA_3) positivity of spermatozoa with some sperm parameters and in vitro fertilization rate.*
Methods: *Spermatozoa were collected from 139 men, washed in PBS, fixed in methanol/glacial acetic acid (3:1), and then spread on slides. CMA_3 positivity is expressed as the percentage in 200 spermatozoa.*
Results: *Percentage of CMA_3 positivity showed not only a negative correlation with fertilization rate but also a significant difference between fertilizing and nonfertilizing patients. Moreover, percentage of CMA_3-positive spermatozoa showed a negative correlation with count and percentage motility and a positive correlation with percentage of abnormal morphology. Percentage of CMA_3 positivity also had a positive correlation with some abnormalities of head such as amorphous and macrocephaly. Ultrastructural study showed chromatin unpackaging in high CMA_3-positive semen samples in comparison with low CMA_3-positive semen samples.*
Conclusion: *There is a close relationship among fertilization rate, sperm parameters, and CMA_3 positivity and CMA_3 could be considered as a useful tool for evaluation of male fertility prior to infertility treatment.*

KEY WORDS: chromomycin A_3; fertilization; IVF; spermatozoa.

INTRODUCTION

Semen quality is conventionally determined according to the number, motility, and morphology of spermatozoa in an ejaculate (1). In turn, it is generally accepted that an association exists between these semen parameters and fertilizing ability (2–4).

However, a failure of the conventional semen parameters to predict fertilization indicates that hidden anomalies lie at the sperm membrane level or at the chromatin level. A number of studies have shown that spermatozoa with abnormal nuclear chromatin organization are more frequent in infertile men than in fertile men (5–7). In this respect many sperm functional tests for sperm chromatin have been developed in order to evaluate the relation between chromatin status and the fertilizing ability of a semen sample (8–10). One of these tests includes the use of chromomycin A_3 (CMA_3) (10).

CMA_3 is a flurochrome which has been shown to compete with the protamines for binding to the minor groove of DNA (10). During spermiogenesis DNA histones are mainly replaced by protamines in order to acquire a highly packed sperm chromatin structure (11).

Therefore CMA_3 represents a useful tool for assessing the packaging quality of the chromatin in sperm and may allow indirect visualization of protamine deficiency.

There are some reports on CMA_3 and its relation to sperm fertilization ability in in vitro fertilization (IVF), subzonal insemination (SUZI), and intracytoplasmic sperm injection (ICSI) (10–13). In ICSI cases Sakkas *et al.* (13) reported that the percentage CMA_3 positivity does not postulate failure of fertilization entirely and suggested that poor chromatin packaging contributes to a failure in the decondensation process and probably reduces fertility in ICSI cases. Shoukir *et al.* (14) reported that abnormal morphology of sperm may manifest a negative paternal effect on preimplantation embryo development, and they suggested that defective protamine packaging in sperm DNA and their

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incorrect replacement by histones during fertilization may also create problems such as asynchronous cleavage and delays in the cell cycle after fertilization, thereby lowering blastocyst formation.

Bianchi (10) showed a positive correlation between fertilization failure and CMA_3 fluorescency in SUZI treatment group. However unlike Lolis *et al.* (12), who showed a close relationship between CMA_3 staining and fertilization in IVF, Bianchi did not observe any correlation between CMA_3 staining and in vitro fertilization. The difference between Bianchi and Lolis could be due to different patient samples and different evaluation methods.

Thus the aim of this study was to evaluate the relation between CMA_3 and fertilization ability of semen in a larger patient sample and also to see if there is any correlation between CMA_3 and sperm chromatin morphology at the ultrastructural level and other semen parameters.

MATERIALS AND METHODS

Subjects and Treatment

One hundred thirty-nine couples referred to Isfahan Fertility & Infertility Centre were included in this prospective study. Sperm samples were collected by masturbation after 3–4 days of abstinence on the day of oocyte recovery. The greater part of semen was prepared for routine IVF, using a discontinuous (50, 70, 90%) Percoll gradient. The remaining semen was used for sperm assessment and chromomycin staining.

Follicular growth was stimulated in the female partner using a GnRH agonist in combination with human menopausal gonadotropin (hMG). The response to treatment was monitored daily from day 7 of the administration of hMG by ultrasonography. Ovulation was triggered by the administration of 10,000 IU human chrionic gonadotropin (hCG). Oocytes were collected 34–36 hr post hCG, using a simple lumen aspiration needle (CCD, France).

Sperm Assessment

Sperm count was performed in a Makler counting chamber after immobilizing the cells with distilled water (count is expressed as millions/ml). Motility was evaluated by direct microscopic examination, and morphology by the Pappanicolaou staining technique (15). Motility is expressed as a percentage of rapid and/or progressive spermatozoa.

In addition to assessing the percentage abnormal morphology, sperm with abnormal heads were subgrouped as macrocephalic, amorphous, elongate, pear-shape, double head, microcephalic, pin head, and round head.

Chromomycin Staining

To prepare slides for CMA_3 staining (10) the semen aliqout was washed in Dulbecoo's Ca–Mg-free phosphate-buffered saline (PBS) and centrifuged at 1200 rpm for 10 min.

The spermatozoa were washed again, fixed in methanol/glacial acetic acid (3:1) at 4°C for 5 min, and then spread on slides. For CMA_3 (Sigma) staining, each slide was treated for 20 min with 100 μl of CMA_3 solution (0.25 mg/ml in McIlvaine buffer, pH 7.0, containing 10 m*M* $Mgcl_2$). The slides were then rinsed in buffer and mounted with buffered glycerol. Microscopic analysis of the slides was performed on a fluorescent Zeiss axioplane microscope (Germany) with the apporpriate filters. Positively spermatozoa are expressed as the percentage in a count of 200. CMA_3 staining also was performed on samples obtained from insemination medium after discontinuous Percoll processing.

In Vitro Fertilization

Oocytes were collected by an ultrasound-laparoscopic method, isolated in Nunc dishes containing 1 ml IVF medium (Ham's F-10), and kept at 37°C under 5% CO_2. After 3–5 hr they were inseminated using 50,000–100,000 motile spermatozoa per oocyte. Fertilization was assessed under an Olympus stereomicroscope (Japan) by the presence of pronuclei 17–19 hr after insemination.

Electron Microscopy

For electron microscopic study, semen samples were collected from five patients with high CMA_3 positivity and five patients with low CMA_3 positivity. These samples were fixed in glutaraldehyde (2.5%) buffered with PBS (pH 7.2). The fixed semen was then postfixed in osmium tetroxide (1%), dehydrated in graded alcohol solutions, and embedded in Taab embeding resin (England). Ultrathin sections cut with an ultramicrotome Leica (Austria) were collected in copper grides, stained with uranyl-acetate and lead citrate, observed, and sometimes photographed with a Zeiss electron microscope (Germany).

Statistical Methods

All statistical calculations were performed using statistical package for social studies (SPSS Inc., Chicago, IL) software. The positively stained spermatozoa are expressed as the percentage in a count of 200. Correlation coefficients were obtained and linear regression analysis was used to evaluate correlation coefficients and statistical significance. Student's *t* test was used for the calculation of significance of values between fertilizing and nonfertilizing groups. The relationships between fertilization rate and all sperm tests were examined by logistic regression analysis.

RESULTS

CMA_3 Positivity in Relation to Sperm Concentration, Motility, and Morphology

A substantial variation in the percentage of CMA_3-stained cells was observed in ejaculated human spermatozoa, varing between 6.2 and 70.2%. Correlation of these percentages with individual sperm parameters (Table I) showed a positive correlation between percentage of CMA_3-positive spermatozoa and percentage of abnormal morphology and abnormality of head and a negative correlation with count and percentage of motility.

In addition to the significant correlation between percentage of abnormal sperm heads and CMA_3 positivity, percentage amorphous and macrocephalic and the sum of these two parameters had a positive correlation with the percentage CMA_3-positive spermatozoa.

The mean percentage CMA_3 positivity showed a significant difference ($P < 0.001$) between net semen samples (mean = 26.33) and insemination medium samples (Percoll processed) (mean = 23.21).

Table I. Results of Statistical Analysis of Correlation Between CMA_3 Positivity and Other Sperm Parameters

Variable	*r*	Significance (*P*-value)
Count ($\times 10^6$/ml)	−0.176	0.049
Motility (%)	−0.281	0.001
Abnormal morphology (%)	0.700	<0.001
Abnormal head (%)	0.698	<0.001
Amorphous head (%)	0.587	<0.001
Macrocephalic head (%)	0.621	<0.001
Macrocephalic + Amorphous head (%)	0.726	<0.001

CMA_3 Positivity and Other Sperm Parameters in Relation to Fertilization Rate

There was a positive correlation between percentage CMA_3 positivity and in vitro fertilization. Moreover, percentage of abnormal morphology, abnormality of head, amorphous head, macrocephalic head, and the sum of these two parameters had a strong negative correlation with IVF (Table II).

Dividing patients into fertilizing and nonfertilizing groups, the mean of count, male and female ages, oocyte number, and percentage double, pin, and round head sperms were similar in two groups. Significant differences were observed, between the two groups, in the other parameters (Table III). Moreover, the mean percentage of CMA_3-positive spermatozoa between fertilizing and nonfertilizing groups had significant differences.

To determine which groups of sperm characteristics were independently related to fertilization rates, all data were examined by logistic regression analysis. With the use of this model the percentage abnormality ($P < 0.001$) and the percentage of CMA_3-positive spermatozoa in insemination medium (Percoll processed) ($P < 0.001$) were independently related to fertilization rates, however, the percentage abnormality was the factor most related to fertilization rate since it was first entered into the equation and its log likelihood was higher than the CMA_3 positivity.

As mentioned above, the mean percentage of double, pin, and round head spermatozoa were similar in fertilizing and nonfertilizing groups but the mean percentages of amorphous, macrocephalic, macrocephalic + amorphous, pear shape, elongated, and microcephalic heads were different in the two groups (Table III). Using paired *t*- test, no statistical difference was

Table II. Results of Statistical of Correlation Between Fertilization Rate and Sperm Parameters

Variable	*r*	Significance (*P* value)
Count ($\times 10^6$/ml)	0.325	0.704
Motility (%)	0.246	0.003
Abnormal morphology (%)	−0.899	<0.001
Abnormal head (%)	−0.845	<0.001
Amorphous head (%)	−0.599	<0.001
Macrocephalic head (%)	−0.605	<0.001
Macrocephalic + amorphous head (%)	−0.714	<0.001
CMA_3 positivity (%) Semen Percoll processed	−0.641	<0.001
	−0.622	<0.001

Table III. Results of IVF in Relation to Some Sperm Parameters

	Mean ± SD		
	Fertilizing (>0) (N = 119)	Nonfertilizing (=0) (N = 20)	Significance (P value)
Count ($\times 10^6$/ml)	79.91 ± 42.70	70.95 ± 47.46	0.997
Motility (%)	51.50 ± 14.39	44.00 ± 14.65	0.033
Abnormal morphology (%)	52.05 ± 16.78	85.00 ± 7.41	<0.001
Abnormal head (%)	44.62 ± 16.69	77.40 ± 13.39	<0.001
Amorphous head (%)	11.13 ± 6.80	22.65 ± 11.47	<0.001
Macrocephalic head (%)	11.8 ± 7.53	19.95 ± 7.77	<0.001
Macrocephalic + amorphous head (%)	22.20 ± 12.06	42.60 ± 12.48	<0.001
Pear-shape head (%)	6.30 ± 5.03	10.15 ± 6.17	0.003
Elongate head (%)	4.05 ± 4.22	8.20 ± 5.99	0.007
Microcephalic head (%)	9.35 ± 6.20	13.50 ± 6.21	0.006
CMA_3 positivity(%) Semen	25.57 ± 11.75	37.56 ± 15.18	<0.001
Percoll processed	21.40 ± 11.49	33.94 ± 13.29	<0.001

obtained between the duplicate reading of slides (one observer) for morphology ($P = 0.77$) and CMA_3 positivity ($P = 0.74$). Also, there were no statistical differences between the means of values obtained for morphology ($P = 0.44$) and CMA_3 positivity ($P = 0.58$) when the same slides were read by two observer.

Ultrastructural Study

Samples with a high positivity for CMA_3 showed incomplete condensation of chromatin and nuclei that displayed a coarsely granular aggregated pattern which is typical of late spermatids (Fig. 2). In contrast, sam-

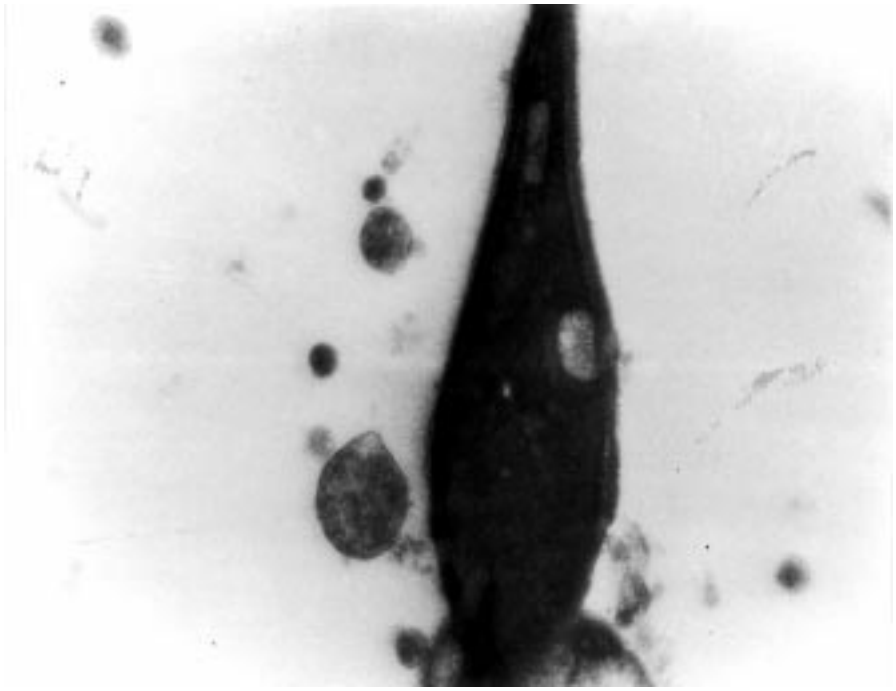

Fig. 1. Section of a spermatozoon in a patient belonging to the group with low CMA_3 positivity. Note the highly compacted aggregation of uniformly dense chromatin ×12,000.

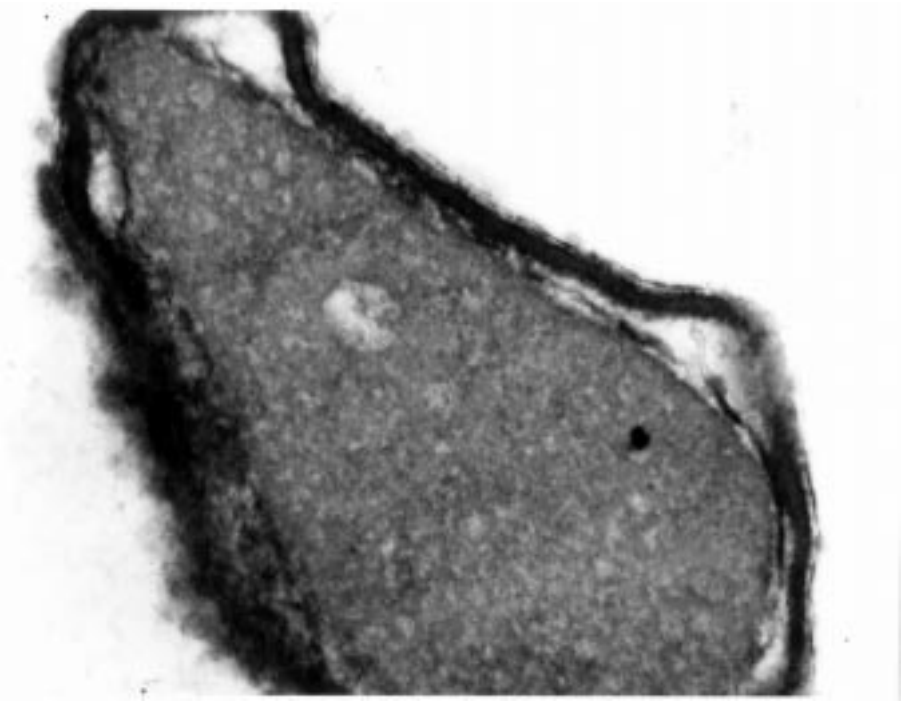

Fig. 2. Section of a spermatozoon in a patient belonging to the group with high CMA_3 positivity. Note the chromatin immaturity of the nucleus. ×20,000.

ples in the low positivity group showed a normal pattern of chromatin packing (Fig. 1).

Also, evaluating samples with a high CMA_3 positivity (>45%) showed that 50 to 70% of spermatozoa had a coarsely granular aggregation pattern of chromatin, however, in samples with a low CMA_3 positivity (<15%), 5 to 10% of spermatozoa showed this pattern.

DISCUSSION

Sperm morphology has a profound effect on fertilization (4,16) and normal embryonic development (14). Normal spermiogenesis is required for formation of normal spermatozoa.

One of the central events of spermiogenesis is the substituation of the chromatin proteins (especially histones) by protamines, allowing a different structural organization to take place in the sperm nucleus. This type of organization provides sperm with a highly condensed nucleus (11) and also protects sperm DNA against the enzymatic attack of nucleases and polymerases. 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 nucleus and influence sperm quality (morphology) and fertilizing capacity.

Numerous groups have shown that male-factor infertility patients possess hidden anomalies in the components of their sperm nuclei, displaying higher levels of loosely packaged chromatin and damaged DNA (5,7,17).

In some studies CMA_3 fluorochrome has been used to evaluate sperm quality and fertilization capacity. The results obtained from these studies are contradictory. Our results showed a negative correlation between percentage CMA_3 positivity and in vitro fertilization. Percentage CMA_3 also had a positive correlation in relation to percentage abnormality and a negative correlation with motility and sperm concentration.

Our results are in accordance with those of Lolis *et al.* (12), who compared the mean percentage of CMA_3 positivity between fertilizing and nonfertilizing groups. However, results reported by Lolis *et al.* (12) and our results, which compare not only the relation between percentage CMA_3 positivity and fertilization rate, but also mean percentage CMA_3 in fertilizing and nonfertilizing groups, differ from those reported by Bianchi *et al.* (10), who suggested that there is no association between CMA_3 fluorescence and fertilization ability for patients being treated by routine IVF. These differences could be due to different patient selections and number of samples.

Fertilization is considered a multifactorial process (many factors could affect fertilization dependently or independently). In order to see which factors affect fertilization rate independently, our data were examined by a logistic regression analysis suggested by Liu *et al.* (4,16). The results of this analysis suggest that even though many factors were correlated with fertilization rate, percentage abnormal morphology and CMA_3 positivity after Percoll processing independently affect fertilization rate. Therefore one may conclude that it is probable that anomalies in spermiogenesis during protamination render spermatozoa functionally immature and thus the deficient chromatin structure may limit the fertilization of oocytes by spermatozoa. These results also suggest that the high CMA_3-positive spermatozoa in an insemination sample may compete with low CMA_3-positive sperm in that sample and thereby reduce the fertilization rate. Therefore processing of the semen sample decreases the percentage of CMA_3-positive spermatozoa in comparison with those in the net semen.

To this day, sperm quality has been conventionally judged by parameters such as concentration, motility, and morphology. Furthermore, many authors have concentrated their attention on the predictive value of morphology (3,18), and it is felt that a normal morphology is indicative of normal function, while high levels of morphologically abnormal spermatozoa in the ejaculate are associated with a reduced fertilization potential (19). In our study percentage abnormality had the strongest negative correlation with IVF rate. Moreover, by categorizing sperm head morphology, our data showed that amorphous and macrocephalic and the sum of these two categories were the groups most related to IVF rate. It is interesting to note that there was a strong positive correlation between CMA_3 percentage and percentage abnormality, which possibly suggests that sperm protamine deficiency leads to abnormal sperm morphogenesis. High CMA_3 positivity was observed especially in samples containing high macrocephalic or amorphous spermatozoa. These results are in accordance with those of Bianchi *et al.* (10), who reported in IVF patients that than 75% of the spermatozoa displaying macrocephaly fluoresced brightly with CMA_3, while almost 50% of spermatozoa classed as having an amophous head shape fluoresced. Lolis *et al.* (12) also suggested that samples with excessive morphological anomalies were strongly correlated with $>60\%$ staining for CMA_3. Bianchi *et al.* (10) and Lolis *et al.* (12) studied the relation between CMA_3 positivity and concentration and motility of spermatozoa. We also studied it, and our results were in accordance with those found by them, which means that CMA_3 positivity increases with reduced sperm concentration and motility. The results of our ultrastructural study demonstrate that protamine deficiency has apparent effects on the sperm nucleus, and high CMA_3 positivity could be a sign of ultrastructural changes of sperm nuclei. The results of CMA_3 staining and ultrastructural study of high CMA_3 positive samples suggest that CMA_3 could become a useful tool for evaluation of male-factor patients prior to infertility treatmant.

CONCLUSIONS

Our results show that CMA_3 has a relation with (1) in vitro fertilization, (2) sperm concentration, (3) sperm motility, and (4) sperm morphology. Since CMA_3 positivity reflects protamine deficiency, deficiency of protamine not only decreases the fertilization rate but also affects other sperm parameters, especially sperm morphology, which is the factor most related to fertilization rate.

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