Original Article

# Credibility of Chromomycin A3 Staining in Prediction of Fertility

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Abstract

**Background:** Chromomycin A3 (CMA3) staining has been used to assess protamine deficiency. The aim of this study was to determine credibility of CMA3 along with semen parameters for assessment of fertility potential.

**Materials and Methods:** Semen analysis and CMA3 staining were carried out on 234 fertile and 178 subfertile individuals. Semen analysis was assessed according to WHO criteria. Protamine deficiency was assessed by CMA3 staining.

**Results:** Means, range of variables, coefficients of correlation and receiver operating characteristic (ROC) analyses of semen parameters and protamine deficiency were determined. Mean values of three main sperm parameters and the percentage of sperm with negative CMA3 were significantly different between fertile and sub fertile groups. The results of CMA3 assessment showed significant correlation with sperm density, percentage of motility and normal morphology in the total population, while in the subfertile group the results of CMA3 showed significant correlation with sperm density and normal morphology. However in fertile men, the only significant correlation was observed between sperm with negative CMA3 and normal morphology. ROC analyses revealed that CMA3 staining has a higher potential to predict fertility status, compared to semen parameters.

**Conclusion:** Assessment of protamine deficiency could be considered as one of the complementary tests along with semen analysis for assessment of fertility.

***Keywords:*** Chromomycin A3, Morphology, Protamine, Motility, Semen Analysis



## Introduction

Approximately 15% of couples are sub-fertile and, in half of them, male factors are involved (1). Semen analysis is routinely considered as the first step in evaluation of male fertility. Ideally, semen quality should predict the fertility potential in an individual. Several reports describe differences in semen quality between fertile and subfertile groups. But recent studies suggest that the determined cut off values by WHO are not sufficient to label fertility status in men throughout the world (2-4) and these values differ in each region (5-7). Therefore, it seems each region needs its own criteria, in order to evaluate the fertility status of couples.

Furthermore, evaluation of sperm parameters on its own seems to not to be sufficient to determine the fertility status of an individual. Therefore, sperm functional tests have been designed, based on certain characteristics of spermatozoa, to be used along with semen analysis to assess fertility potential (4).

Since chromatin content of sperm approximately contributes to one half of the genomic material of an offspring and its integrity effects fertilization, pre and post implantation development, therefore, assessment of sperm chromatin integrity along with semen analysis may help patient management (8).

Among sperm chromatin related tests much emphasis has been given to the sperm chromatin structure assay (SCSA) test that has been widely used to assess fertility potential of both infertile and fertile individuals and is based on the ability of sperm to undergo DNA deterioration upon heat or acid treatment (9, 10). Chromomycin A3 (CMA3) has also been used to assess chromatin status of sperm (11, 12). *In situ* CMA3 competes with protamine, the major protein involved in DNA packaging in sperm. Thus, the degree of CMA3 staining inversely correlates with the protamination state and indirectly assesses protamine deficiency (12). This test is simple and easily performed in andrology labo-

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ratories (13). Protamine deficiency is also related to ultra structural anomalies of sperm chromatin (14). Interestingly, the percentage of CMA3 that stains positive has been shown to be increased in the sperm cells of infertile patients (15, 16). Correlations between CMA3 staining and assisted reproduction outcomes have also been reported (8, 12).

Furthermore, one of the mechanisms underlying failed fertilization in assisted reproductive techniques, such as intra cytoplasmic sperm insemination (ICSI), has been related to protamine deficiency (16-19). Even though the significance of protamine deficiency in assessment of male infertility is evident, however, there are no literature studies available on assessment and comparison of protamine deficiency between fertile and infertile groups. Therefore, the aim of this study is to determine the cut off values of semen parameters in our region based on the previous studies and to evaluate the potential and credibility of CMA3 staining, for assessment of protamine deficiency to predict the status of fertility.

## Materials and Methods

This study was initially approved by the Ethical and Scientific Committee of Isfahan Fertility and Infertility Center and Royan Institute. Semen samples were obtained from 234 fertile men and 178 sub-fertile controls. Cases were selected according to studies carried out by Ombelet et al. (20). They evaluated the semen parameters of semen samples obtained from the partners of pregnant women and evaluated the lower 10th percentile of each parameter as the cut off value. In this report, they also analyzed the ROC curve to evaluate sensitivity and specificity of each semen parameter from both fertile and sub fertile couples by using fertility status as the golden standard (20, 21). Thus, semen samples were obtained from the partners of fertile men whose partners were pregnant at the time of study. These couples did not have a history of previous sub fertility or habitual abortion. All men volunteered without payment and after consultation, a consent form was signed. For the sub fertile control group, during the study period we examined 200 consecutive couples who attended the Isfahan Fertility and Infertility Center for the first time with a history of sub-fertility for at least 13 months. The semen samples were collected by masturbation after 2-7 days of abstinence (4, 22). Routine semen analysis was carried out by light microscopy according to WHO criteria (2). Semen samples with lower than 3 million per ml were excluded from the study since adequate numbers of sperm were required for assessment of semen parameters and CMA3 staining. Analyses were carried out by a single individual blinded to the fertility status of the study subjects.

### *Sperm preparation*

A portion of the semen samples was washed with 5ml of HAM'S F10 + 10% HSA (Human Serum Albumin) at 2000 rpm. The prepared samples were used for evaluation of sperm morphology and protamine deficiency using CMA3 staining and Papanicolaou staining, respectively (12, 23).

### *Assessment of sperm morphology (Papanicolaou staining)*

Washed semen samples were stained by the modified Papanicolaou technique. Sperm morphology was assessed according to WHO (2). The analysis of sperm morphology included an assessment of the sperm head, neck, tail and immature cytoplasmic remnant. Spermatozoa were considered normal when the head had a smooth oval configuration with a well defined acrosome involving about 40-70% of the sperm head, as well as an absence of neck, mid-piece or tail defects. No cytoplasmic droplets of more than one third the size of the sperm head were accepted.

### *Assessment of protamine deficiency (Chromomycin A3 staining)*

Washed semen samples were fixed in Carnoy's solution [methanol: glacial acetic acid 3:1 (Merck, Germany)] at 4°C for 5 minutes. Smears were prepared and each slide was treated for 20 minutes with 100 µl of CMA3 solution (Sigma, USA) [500 µg/ml in McIlvaine buffer (7 ml citric acid 0.1 M + 32.9 ml $Na_2HPO_4.7H_2O$, 0.2 M, pH 7.0, containing 10 mM $MgCl_2$)]. Then, the slides were rinsed in buffer and mounted with buffered glycerol (1:1). Microscopic analysis of the slides was performed on an Olympus fluorescent microscope (BX51, Tokyo, Japan) with the appropriate filters (460–470 nm). On each slides, 200 sperm cells were examined. Evaluation of CMA3 positivity was carried out using Olysia software. Pixel intensity of each sperm was recorded. Sperm with a pixel intensity of higher than 100 was considered as CMA3 positive or protamine deficient, while those with a pixel intensity of lower than 100 were considered as CMA3 negative (Fig 1).

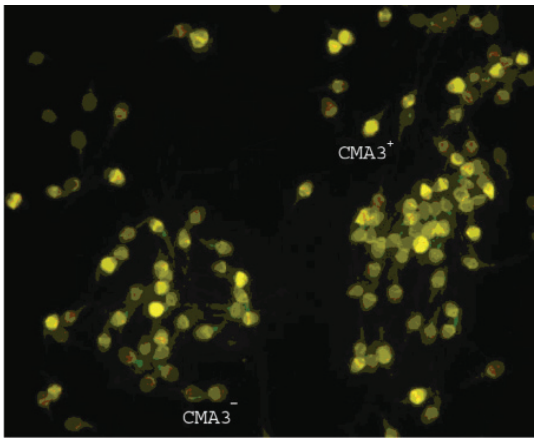


*Fig 1: Chromomycin A3 (CMA3) staining, CMA3 negative or the spermatozoa with normal protamine content appear dull, while CMA3 positive or protamine deficient spermatozoa appear as bright.*

Data in the tables were expressed as % CMA3 negative. Analysis of coefficient of variation using Olysia software reveals that this method has a lower intra-assay variation (data not shown) than the conventional method (12).

### *Statistical analysis*

Kolmogorov-Smirnov Z test was used to assess the normal distribution of data. Mean, range of variables, coefficients of correlation and student t-test were carried out using the Statistical Package for the Social Studies (SPSS 11.5; Chicago, IL) software to compare results between different groups.

## Results

Table 1 illustrates descriptive statistics of semen parameters including:sperm density, percentage motility; normal morphology; and negative CMA3 in sub-fertile, fertile couples and total samples which were evaluated in this study.

The results in Table 1 show that the mean values of semen parameters and percentage sperm with negative CMA3 are significantly different between fertile and sub fertile groups (Table 1).

The results in Table 2 illustrate the correlations between semen parameters and percentage of sperm with negative CMA3 among fertile, sub fertile groups and total samples. In all groups, semen parameters showed significant correlation with each other. The percentage of sperm with negative CMA3 shows significant correlation with density only in sub fertile and total samples. This parameter also showed significant correlation with motility only in the total samples and with normal morphology in the three groups.

*Table 1: The mean value, standard deviation, range of each semen parameter and percentage of sperm with negative CMA3 in fertile, sub fertile groups and total samples.*

| Parameters | Fertile group | | Sub fertile group | | P value | Total samples | |
|---|---|---|---|---|---|---|---|
| Variables | Range | Mean ± SD | Range | Mean ± SD | | Range | Mean ± SD |
| Density (×10⁶/ml) | 15-368 | 136.33 ± 80.04 | 3-366 | 111.31 ± 82.77 | 0.000 | 3-368 | 125.59 ± 82.06 |
| % Motility | 15-98 | 75.59 ±13.17 | 0-95 | 60.57 ± 20.30 | 0.000 | 0-98 | 69.15 ± 18.18 |
| % Normal morphology | 10-96 | 49.75 ± 16.23 | 0-70 | 29.13 ± 18.82 | 0.000 | 0-96 | 40.90 ± 20.15 |
| % Negative CMA3 | 39-100 | 85.65 ± 12.59 | 4-100 | 52.18 ± 26.60 | 0.000 | 4-100 | 71.26 ± 25.84 |

*Table 2: Correlation coefficients between semen parameters and percentage of sperm with negative CMA3 in three groups (fertile, sub-fertile groups and total samples).*

| Parameters | Normal Morphology (%) | | | Motility (%) | | | Density (million/ml) | | |
|---|---|---|---|---|---|---|---|---|---|
| Variables | Fertile | Sub-fertile | Total | Fertile | Sub-fertile | Total | Fertile | Sub-fertile | Total |
| Density (×106/ml) | 0.285** 0.000 | 0.470** 0.000 | 0.397** 0.000 | 0.218** 0.001 | 0.524** 0.000 | 0.407** 0.000 | 1 | 1 | 1 |
| % Motility | 0.244** 0.000 | 0.408** 0.000 | 0.475** 0.000 | 1 | 1 | 1 | 0.218** 0.001 | 0.524** 0.000 | 0.407** 0.000 |
| % Normal morphology | 1 | 1 | 1 | 0.244** 0.000 | 0.408** 0.000 | 0.475** 0.000 | 0.285** 0.000 | 0.470** 0.000 | 0.397** 0.000 |
| % Negative CMA3 | 0.135* 0.040 | 0.219** 0.003 | 0.448** 0.000 | -0.044 0.505 | 0.068 0.370 | 0.291** 0.000 | 0.057 0.382 | 0.219** 0.003 | 0.215** 0.000 |

**Correlation is significant at the 0.05 level (2-tailed).*
***Correlation is significant at the 0.01 level (2-tailed).*

*Table 3: The area under curve using receiving operative characteristic (ROC) curve analyses for density, motility, normal morphology and percentage with sperm negative CMA3. Fertility considered as state variable or gold standard.*

| Parameters | Area under curve | Standard Error | 95% Confidence Interval | | Cut off value | Sensitivity | Specificity |
|---|---|---|---|---|---|---|---|
| | | | Lower Bound | Upper Bound | | | |
| **Density** | 0.604 | 0.028 | 0.549 | 0.660 | 47.5 | 885 | 0.742 |
| **Motility** | 0.733 | 0.025 | 0.683 | 0.783 | 56.5 | 0.902 | 0.646 |
| **%Normal morphology** | 0.790 | 0.022 | 0.746 | 0.834 | 33.5 | 0.842 | 0.393 |
| **% Negative CMA3** | 0.855 | 0.020 | 0.816 | 0.893 | 39 | 1.000 | 0.640 |

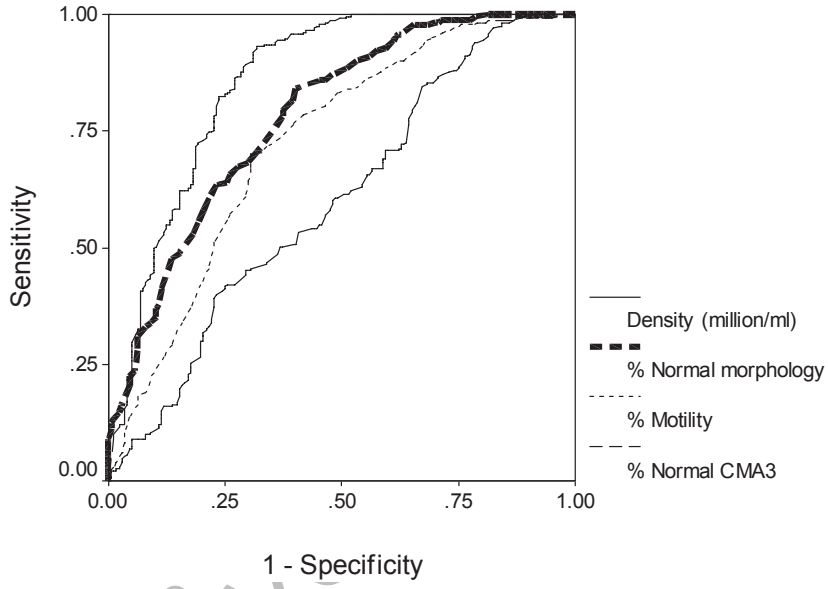


*Fig 2: Receiver operating characteristic (ROC) curve for the semen parameters and percentage of sperm with negative CMA3.*

Table 3 demonstrates the result of receiver operating characteristic (ROC) curve analysis using fertility as state variable or gold standard (Fig 2).
As this table shows, the area under the curve in all three semen parameters and percentage of sperm with negative CMA3 were higher than 0.5 and were significant.
Among the four parameters, the area under the curve for the percentage of sperm with negative CMA3 is greater than the three semen parameters. Therefore, these results show a higher specificity and sensitivity of CMA3 staining in prediction of fertility status. Also this table shows the 95% confidence intervals for the upper and lower bounds which were above 0.5. The cut off values of density, motility, normal morphology and percentage of sperm with negative CMA3 are 47.5, 56.5, 33.5 and 39, respectively. Considering a round cut of values for CMA3 negative sperm as 40% and for normal morphology as 30%, except one individual, 99.6% in the fertile group had CMA3 negative sperm higher than 40%; while 85.5% had normal morphology of greater than 30%, the cut off value which is close to that of WHO.

## Discussion

The role of traditional semen analysis and semen parameters as a prognostic factor of male fertility potential is a matter of on-going debate (20, 23-25). Especially for the *in vivo* situation, there is a lack of information on normal and minimal values on sperm concentration, sperm motility and morphology. The results of this study (Table 1) reveal that the mean values of sperm density and motility in the fertile group were $136\times10^6$/ml and 75%, which appear to be different from reported ranges of mean values. The range of mean values which previously have been reported for sperm density was $53\times10^6$ -$104\times10^6$ /ml and for motility was 53%-67%, respectively (2, 20, 21, 25). The percentage of normal sperm morphology in this study was 49% which is within the range and comparable with the other studies (21).
Table 1 also indicates that the mean values of the three mentioned main semen parameters and the percentage of sperm with negative CMA3 are significantly different between fertile and sub fertile groups. Therefore, this data may suggest that

CMA3 values might have a diagnostic value along with the semen parameters.

The results of this study show significant correlation between the three main semen parameters in fertile, sub fertile groups and the total population. Thus, suggesting that events taking place during spermatogenesis are related to each other and are not independent. These correlation coefficients were higher in the sub fertile group compared with the fertile group (Table 2). This difference is possibly due to a wider range of variables or greater heterogeneity in the sub fertile group (Table 1).

The percentage of sperm with negative CMA3 shows significant correlation with three main sperm parameters in the total samples. However, in the fertile group, the percentage of sperm with negative CMA3 is only correlated with normal morphology and in the sub fertile group, the percentage of sperm with negative CMA3 show significant correlations with sperm density and normal morphology. Thus, suggesting that in sub-fertile or infertile cases with sever oligospermia, there is a higher degree of protamine deficiency.

Fig 2 reveals the ROC analyses of three main semen parameters and percentage sperm with negative CMA3 using fertility as state variable or golden standard. The area under the curve is above 0.5 and increases from density, motility and normal morphology to percentage sperm with negative CMA3. Furthermore both upper and lower bounds of the 95% confidence interval in all parameters are higher than 0.5, which suggest that these parameters have diagnostic value. The area under the curves also indicate firstly the percentage of sperm with negative CMA3 and then sperm normal morphology have a better diagnostic value compared with sperm motility and density. The results of ROC analyses of normal morphology in consistent wich has higher ability for prediction of fertility than the two other main sperm parameters (12, 26). However, the ROC analysis of percentage sperm with negative CMA3 shows that evaluation of CMA3 could be a better tool for prediction of fertility status than the normal morphology. The cut off values of semen parameters and percentage sperm with negative CMA3 suggest that semen samples with density values higher than $47.5\times10^6$/ml, 56.5% motility, 33.5% normal morphology and 39% CMA3 negativity have a good chance for fertility, in vivo. By rounding the cut off value of CMA3 to 40% and dividing the individual to fertile and infertile group, 99.6% and 38% of the fertile and sub-fertile groups are considered as fertile, respectively. However, when taking the WHO criteria of normal morphology as the cut off value; 85.8% and 46 % the fertile and sub-fertile group are considered as fertile, respectively. The difference may suggest that assessment of CMA3 may complement the diagnostic values of semen parameters in evaluation of male infertility.

In vitro studies on IVF candidates illustrated that sperm normal morphology and sperm with negative CMA3 have a good correlation with fertilization rate (8, 27). However, in ICSI procedures, unlike CMA3, sperm morphology did not show significant correlation with fertilization rate (17). Some recent studies using special imaging system and assessing sperm morphology of the inseminated sample, revealed that sperm morphology and normality of nucleus have significant effects on fertilization, embryo development, and pregnancy outcome (18, 28, 29). The other studies, using ROC analyses for prediction of fertilization potential post-IVF, reveal that CMA3 has a high sensitivity and specificity value for prediction of fertilization (12, 16). Possible reasons for CMA3 being a useful tool for assessing fertility are: 1) its relation with DNA integrity and sperm maturation (14, 30-33), 2. failed fertilization due to induction of premature chromosomal condensation (34) and 3. lower potential for oocyte activation (35).

## Conclusion

CMA3 staining as an indicator of protamine content, along with semen analysis could be a useful test for evaluating fertility status in sub fertile cases.

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