Original Article

Flow Cytometry: A New Approach for Indirect Assessment of Sperm Protamine Deficiency

Marziyeh Tavalaee, M.Sc.[1], Abbas Kiani, M.Sc.[1], Maryam Arbabian, B.Sc.[1], Mohammad Reza Deemeh, M.Sc.[1, 2], Mohammad Hossein Nasr Esfahani, Ph.D.[1, 2*]

1. Reproduction and Development Department, Royan Institute for Animal Biotchnology, ACECR, Isfahan, Iran
2. Isfahan Fertility and Infertility Center, Isfahan, Iran

Abstract

Background: Flow cytometry (FCM) has been extensively used to study mammalian sperm in the areas of clinical andrology and reproductive toxicology. FCM provides a powerful advantage over microscopy technique in terms of rapid, accurate and reproducible technology for the quantification of various cell characteristics, including chromatin status. During spermiogenesis, histones are replaced by protamines resulting in a very condensed structure of sperm chromatin. Infertile men have an increased sperm histone: protamine ratio than fertile counterparts. Chromomycin A3 (CMA3) staining represents a useful tool for assessing the packaging quality of sperm chromatin and allows indirect visualization of protamine deficiency. Routinely, fluorescence microscope is used for evaluation of protamine deficiency by CMA3. Considering the advantages of FCM and increasing use of CMA3 in assessment of protamine deficiency in the literature and its possible use as a diagnostic test, the aim of this study is to standardize this procedure for routine laboratory analysis.

Materials and Methods: Semen samples were collected from 85 infertile men who referred to Isfahan Fertility and Infertility Center. A portion of semen sample was used for routine semen analysis according to WHO criteria and the remainder were evaluated to standardize CMA3 staining procedure for fixation, the number of sperm and duration of exposure to CMA3. The results were compared with standard fluorescent microscopic procedure. Percentage CMA3 positive sperm were compared between flow cytometry and standard fluorescent microscopic procedure.

Results: Our results show that fixation, the number of sperm and duration of exposure to CMA3 can affect on FCM outcomes. In addition we show that the samples can be fixed, stained with CMA3, stores and then assessed for FCM.

Conclusion: The optimal conditions for FCM assessment of CMA3 are: fixation, concentration of 0.25 mg/ml, sperm density of 2 million/ml and exposure for 60 minutes.

Keywords: Chromomycin A3, Flow Cytometry, Protamine, Andrology, Microscopy Technique

Introduction

Considering the advances in the field of assisted reproduction technology (ART) and implementation of new sperm selection procedures based on sperm DNA integrity, have increased emphasis on sperm chromatin quality. In intracytoplasmic sperm injection (ICSI), an apparently "normal" sperm is selected and used for insemination. Therefore, during this process, the most of natural selection barriers are bypassed and therefore, increasing the possible risk of genetic abnormalities that can have consequence including failed fertilization and embryo development. These consequences become more marked in semen samples with poor quality (1-3). Several factors such as: sperm parameters, acrosome, and chromatin structure have assessed by microscopy as a potential factor to predictor fertility. However, until now, no sole laboratory test on its own can assess fertility potential. As stated by Evenson et al. 1999 disadvantages of microscopy technique are intra-observer variations, low number of spermatozoa analyzed leading resulting in low statistical value (4, 5). Recently computer-interfaced flow cytometry (FCM) has entered the andrology laboratory and several studies had used from this technique for evaluation of chromatin structure (6), acrosomal status (7), spermatogenetic defects and etc (4, 8). The advantage of FCM includes; rapid, accurate, objectivity, reproducible and power statistical analysis over microscopy

Received: 12 Oct 2009, Accepted: 19 Dec 2009
*** Corresponding Address: Reproduction and Development Department, Royan Institute for Animal Biotchnology, ACER, Isfahan, Iran**
Email: mh.nasr-esfahani@royaninstitute.org

Royan Institue
International Journal of Fertility and Sterility
Vol 3, No 4, Feb-Mar 2010, Pages: 177-184

techniques. In addition, FCM can sort cells based on different cellular characteristic which may have application in assisted reproduction techniques such as sperm sexing (4, 9, 10).Therefore, FCM can be used in clinical setting in order to evaluate fertilization potential in andrology laboratories.

Integrity of sperm chromatin structure has a paramount effect on ICSI outcome. Therefore, multiple assays have been developed to measure sperm chromosomal aberrations, abnormal chromatin packaging and chromatin structural integrity by using FCM (11). The chromatin structure of the sperm is very different from that of somatic cells. During spermiogenesis, histones are replaced first by transition proteins followed by protamines resulting in a very condensed structure of sperm DNA which is further stabilized by formation of disulfide bridges between some of the thiol group (-SH) of protamines during epididymal transition. Subsequently, non covalent bonds are formed between free thiol groups by prostatic Zn^{2+} (12-14). It has been shown that the protamine content has been altered in infertile men compare to fertile individuals (15). Therefore alteration of chromatin structure can result in abnormal packaging which makes chromatin susceptible to sperm to DNA damage (12, 16).

During routine ICSI, sperm is selected on the basis of morphology and motility which does not guarantee selection of sperm with normal protamine content. Considering the fact that protamine deficiency and sperm DNA damage are related events and result in poor fertilization, analysis of protamine content of semen samples can be of paramount importance in patient management and for assessment of new sperm selection procedures.

The methods used for evaluation of chromatin condensation include: aniline blue staning, SDS+EDTA test, SDS analysis and chromomycin A3 (CMA3) for evaluation of excessive histones, ability of sperm to nuclear decondensation, chromatin stability and indirect measurement of protamine deficiency, respectively (17,18).

CMA3 is a glycosidic antineoplastic antibiotic isolated from the bacterium Streptomyces griseus and reversibly binds to guanine-cytosine (G-C) base pairs in the minor groove of DNA. Therefore, this flurochrome competes with protamine for binding to DNA, which is dependent on magnesium. Therefore, CMA3 identifies spermatozoa with defective packaging and indirectly evaluates protamine deficiency (19, 20). CMA3 has been widely used by researches for assessment of etiology of infertility. There are some reports on CMA3 and its relation to sperm fertilization ability in *in vitro* fertilization (IVF) and ICSI, suggesting that sperm protamine deficiency is associated with fertilization failure (21-23). Recently, CMA3 staining has also implemented for evaluation of bovine sperm (24). Routinely, fluorescence microscope is used for evaluation of protamine deficiency by CMA3. Considering the advantages of FCM and increased usage of CMA3 in assessment of protamine deficiency in the literature, and it's possible use in routine andrology units, the aim of this study is to standardize this procedure for routine laboratory assessment of protamine deficiency.

Materials and Methods

This study received the approval of the Institutional Review Board of Isfahan Fertility and Infertility Center and Royan Institute. Informed consent forms were signed by all patients. All chemicals were obtained from Merck (Germany, Darmstadt), unless otherwise stated.

Sperm Analysis and Sperm Processing

Semen samples were collected from 85 infertile men who referred to Isfahan Fertility and Infertility Center. All semen samples were collected by masturbation into sterile containers after 3-4 days of sexual abstinence and were delivered to the laboratory within 45 min after ejaculation. A portion of semen was used for routine semen analysis according to WHO criteria (25) and the remainder was washed twice in Dulbecco's Ca^{2+}-Mg free phosphate buffered saline (PBS) (pH 7.4). A Semen Analysis Chamber was used for assessment of sperm counts.

Experimental designs

The 85 semen samples were used for the below experimental designs. The number of semen samples used for each experiments are indicated in the parenthesis. Some samples were common between the experiments.

1. To evaluate effect fixation on CMA3 outcomes by FCM and to compare the results with fluorescence microscope analysis (N= 20, Fig 2)
2. To evaluate the effect of number of sperm exposed to fixed volume CMA3 solution on CMA3 outcomes by FCM (N=33, Fig 3A)
3. To evaluate the effect of number of sperm exposed to CMA3 solution on CMA3 outcomes by fluorescence microscopy (N=33, Fig 3B)
4. To define the lowest number of sperm required for FCM (N=10, Fig 3C)
5. To evaluate the effect of duration of exposure to CMA3 on FCM outcome in fixed and unfixed samples (N= 37, Fig 4).

6. To evaluate the time of assessment of CMA3 by FCM post staining (N=21)

Microscopic analysis of CMA3 staining

CMA3 staining was carried out according Bianchi et al. (17) or Iranpour et al. (20). Briefly, semen samples were washed in PBS free Ca^{2+} and Mg^{2+} and were fixed (1:1) in Carnoy's solution at 4°C for 5 minutes. Smears were treated for 20 minutes with 100 µl of CMA3 (Sigma, St Louis, MO, USA) solution. The slides were then rinsed in PBS buffer and mounted. Microscopic analysis of the slides was performed on a fluorescent microscope with the appropriate filters (460-470 nm). On each slide 200 sperm cells were evaluated. Evaluation of CMA3 staining was carried out by distinguishing between spermatozoa with bright yellow staining (CMA3 positive) and spermatozoa with dull yellow staining (CMA3 negative). All microscopic CMA3 analysis was carried out by a trained individual (17, 20).

FCM analysis of CMA3

The flow cytometry-based CMA3 staining assay was adapted from the slide-based method (17). Semen samples were washed with PBS and diluted to appropriate concentration according to experimental design. 1ml of each samples were centrifuged (200 g, 5 minute) and used directly for staining or fixed with Carnoy's solution for 5 minutes at -4°C and then stained. For staining, the samples were centrifuged and the pellets were stained with 200 µL of 0.25 mg/ml CMA3 solution at room temperature. The time of exposure to CMA3 is given for each experimental design. CMA3 solution was prepared as for fluorescence microscopy. Then, samples were washed twice with PBS and assessed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA) using an argon laser with an excitation wave length of 488 nm. Fluorescence from Chromomycin A3 stained sperm was collected in fluorescence detector-2 (FL-2) with a 585/42 nm band pass filter. A minimum of 10000 sperm were examined for each assay and analyzed using WinMDI 2.9 software.

A positive control was obtained by pre-incubating the spermatozoa with 200 mmol dithiothreitol, a disulphide reducing agent, at 37°C for 10 minutes.

Evaluation of fixation on CMA3 outcomes by fluorescence microscope and FCM

In order to determine if fixation affects the outcome, twenty different ejaculate were divided into three equal portions. In each portion was washed with PBS and diluted to 2 million per ml. One portion was fixed while the second portion was used without fixation, then cells were exposed to CMA3 solution for 60 minute and prepared for FCM according to the above procedure. The third portion was used for fluorescence microscope analysis. The results were compared between the three groups.

Evaluation of number of sperm exposed to fixed volume of CMA3 solution on CMA3 outcomes by fluorescence microscopic and FCM

In order to evaluate the effect of number sperm on CMA3 analysis, semen samples from thirty-three infertile individuals were washed, diluted to 2, 4, 8, and 16 million/ml, fixed, centrifuged , exposed to 200 µL of CMA3 solution, for 60 minutes, washed and then assessed by FCM. Concomitantly, in an attempt, to determine whether the number of sperm affects on the CMA3 results by fluorescence microscopic, slides were prepared from the above samples with sperm concentrations of 2 and 20 million/ml.

In order to check the procedural validity for semen samples with very low the number of sperm, ten samples were diluted to 2, 1.5, 1 and 0.5 million/ml. Following fixation and centrifugation, instead of adding 200 µl of CMA3, the volume of CMA3 was reduced proportionally to the number of sperm (200, 150, 100 and 50 µl for 2, 1.5, 1, 0.5 million sperm, respectively) to keep the ratio of sperm density to CMA3 concentration. Following exposure to CMA3 for 60 minutes, sperms were washed and the cells were assessed by flow cytomerty.

Evaluation of duration of exposure to CMA3 on FCM outcome in fixed and unfixed samples

In this study, the effect of the incubation time of CMA3 solution on the percentage of CMA3 positivity was investigated. Therefore thirteen semen samples, each separately diluted to 2 milliom/ml and 6 aliquots of 1ml volume were prepared. Each aliquot was fixed and stained with CMA3 solution as above mentioned, for 20, 40, 60, 120 and 180 minutes and then washed and assessed by flow cytomerty. Similar experiment was repeated on twenty- four unfixed semen samples.

Evaluation of time of assessment of CMA3 by FCM post staining

Since immediate assessment of CMA3 by flow cytometry is not always possible, the aim of this section was to evaluate whether the results of fixed or unfixed and stained samples could be assessed 24 hours later. Therefore, seven semen samples were

fixed, stained with CMA3 for 60 minutes, washed and analyzed immediately or stored for 24 hours at 4°C and then analyzed. Concomitant with the above studies, fluorescence microscope analysis was also performed.

Statistical Analysis

A Kolmogorov-Smirnov Z test was used to assess the normal distribution of data. Coefficients of correlation and Student t tests were carried out using the Statistical Package for the Social Studies (SPSS 11.5, Chicago, IL) software to compare results between different groups. CMA3 values are expressed as mean ± standard error of mean (SEM) and P-value lower than 0.05 was considered as statistically significant.

Results

Fig 1 show the Dot plot of CMA3 staining for spermatozoa. The cells gated in the R1 region were analyzed and debris was excluded from the analysis.

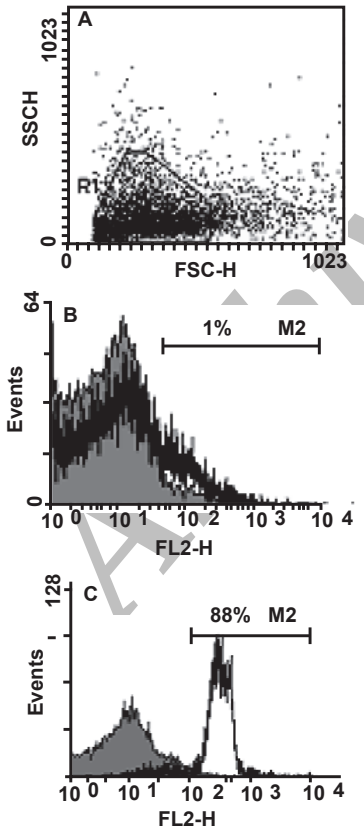

Fig 1: FCM analysis of %CMA3 positive spermatozoa obtained from infertile patients. A: Dot plot of spermatozoa. The cells gated in R1 region were analyzed; debris was excluded from the analysis. B: %CMA3 positivity in semen sample that was not treated with DTT.C: %CMA3 positivity in semen sample that was treated with DTT.

Effect of treatment with DTT

The above semen sample was treated with dithiothreitol (DTT), a disulphide reducing agent. Semen samples were used unfixed and then assessed for CMA3 positivity. The results were compared to the control semen sample. The percent of CMA3 positivity increased from 1% to 88% following treatment with DTT. Fig 1B and 1C showing disulphide reducing agent increases CMA3 positivity.

Effect of Carnoy's fixative on FCM outcome

Fig 2 shows the mean percentages of CMA3 positivity of unfixed and fixed samples by FCM were 43.77 ± 5.00 and 25.03 ± 4.02, respectively. The mean of CMA3 positivity for fluorescence microscope analysis was 44.15 ± 3.1. The mean differences between fixed and unfixed samples for FCM were significantly different ($p<0.01$), while the difference between the unfixed sample with those of the fluorescence microscopic sample were insignificant ($p=0.94$), which suggested that the percentage of CMA3 positivity was reduced following fixation of the samples in FCM. The general trend of CMA3 positivity in the majority of samples was lower in the fixed sample relative to unfixed samples in FCM. However, in some samples, the values of CMA3 in the unfixed samples, assessed by FCM, were not in accordance with fluorescence microscopy results. Therefore, we assessed the coefficient of correlations between these three procedures. The results revealed a significant correlation between fixed with unfixed FCM ($r = 0.632$, $p=0.003$) and between fixed FCM and fluorescence microscopy ($r = 0.336$, $p=0.017$). However, no significant correlation was observed between unfixed FCM and fluorescence microscopy ($r = 0.009$, $p=0.969$).

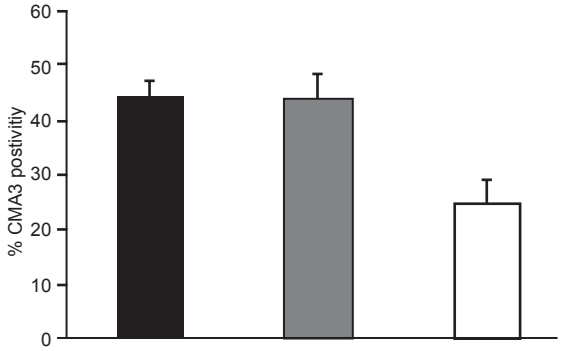

Fig 2: Comparison of percentage of CMA3 positivity by fluorescence microscopy (dark column), fixed (white column) and unfixed (gray column) samples assessed by FCM. The mean value of CMA3 positivity in fixed samples by FCM was significantly different from the other two groups ($p<0.01$). Bars indicate standard error.

Effect of number of sperm exposed to CMA3 solution on CMA3 outcomes by fluorescence microscopic and FCM

Fig 3A shows that the percentage of CMA3 positivity decreased with increased number of sperm exposed to fixed volume of CMA3. The mean CMA3 values of each group is significantly different from the others ($p<0.05$). In addition, the result of fluorescence microscopic evaluation showed no significant difference between low and high density (Fig 3B). Furthermore, the results of figure 3C show no significant difference between the mean CMA3 values when the ratio of number of sperm to volume of CMA3 were maintained. The only significant difference was observed between the mean of CMA3 value for 0.5 million relative to 2 millionsperm exposed to CMA3.

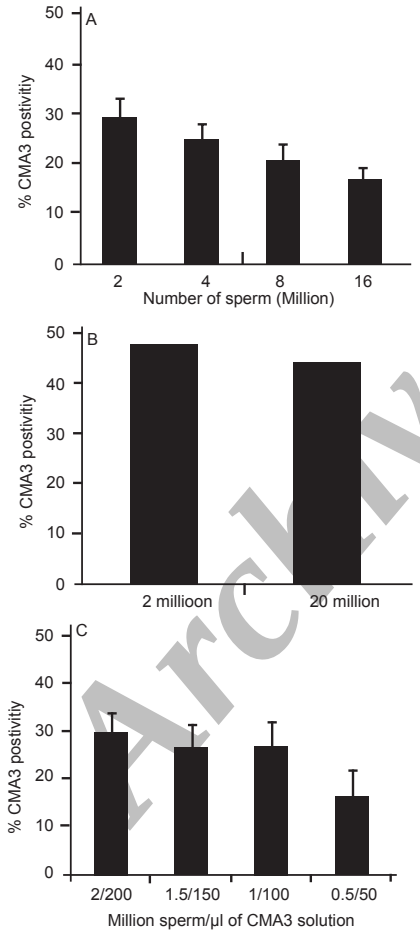

Fig 3: A. Show that number of sperm exposed to fixed volume of CMA3 effects the CMA3 outcome. The mean value of each group is significantly different from the other group at p< 0.05. B. The percentage of CMA3 positivity in fluorescence microscopy for slides prepared with 2 and 20 million/ml were not significant different at P< 0.05. C. Show the mean value of CMA3 positivity in FCM procedure when maintaining the number of sperm to the volume of CMA3 solution during CMA3 staining. Only the last group (0.5 million sperm in 50µL CMA3) was significantly different (p< 0.05) from the first group (2 million sperm in 200 µL CMA3 solution).

Effect of duration of exposure to CMA3 solution on FCM outcome

Fig 4 represents the percentage of CMA3 positivity as a tri-phase pattern in the fixed sample. The percentage of CMA3 positivity increased gradually with time; however it reached a steady state during 40 - 60 minutes with a subsequent increase. Unlike the fixed samples, the percentage CMA3 positivity increases with time in the unfixed sample.

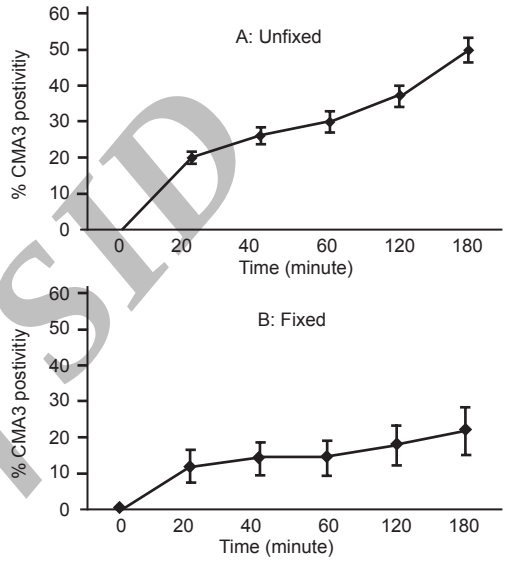

Fig 4: Percentage of CMA3 positivity in the absence (A) or presence (B) of fixative for different exposure periods assessed by FCM.

Effect of the time of assessment of CMA3 by FCM post staining

The results showed no significant difference in FCM analysis between the samples that were stained, washed and assessed immediately or after 24 hours (19.41 ± 7.9 vs 21.58 ± 7.5, p=0.539). Similar results were obtained with another fourteen semen samples that were unfixed, stained and read immediately or read 24 hours later (34.98 ± 4.8 vs 33.70 ± 3.13 p=0.769) . Concomitant with the above studies, fluorescence microscope analysis was also performed. The results showed no significant difference between the samples that were read immediately or 24 hours later (47.42 ± 6.07 vs 40.80 ± 6.30 p=0.449).

Discussion

The importance of sperm chromatin packaging on male infertility has been well demonstrated from transgenes and knockout models for protamine. Proper chromatin packaging, facilitates sperm transport, protects DNA from chemical and physical damage, results in proper gene reprogramming post fertilization, and leads to syn-

chronization of the cell cycle between the oocyte in MII phase and sperm in G1 (26, 27).
Several factors related to sperm nuclear packaging have been identified as clinically significant, including the replacement of nuclear histones with a proper ratio of protamine 1 to protamine 2 (P1/P2 ratio), histone to protamine ratio and the extent of DNA damage in the mature sperm (28, 29). Therefore, measurement of protamine deficiency of human spermatozoa is of particular biomedical interest for diagnosis of male infertility (18). Protamine deficiency can be assessed direct by urea polyacrylamide gel electrophoresis and indirectly by CMA3 staining (21, 30). The latter technique involves fluorescence microscopy and, due to simplicity, it has been widely used by andrologists. However, single cell cytophotometric determinations prove to be time consuming and results can be affected by inter and intra observer variations.
FCM have provided an alternative to the single cell cytophotometric method and have been implemented for sperm since 1970 (31). An as stated by Cordelli et al. "FCM is an automated approach able to measure the amount of one or more fluorescent stains associated with cells in an unbiased manner, offering unmatched properties of precision, sensitivity, accuracy, rapidity and multi-parametric analysis on a statistically relevant number of cells" (2). However, standardization of FCM for growing availability of fluorescent probes is of paramount importance. Therefore, the aim of this study is to standardize CMA3 staining by FCM for the routine andrology laboratory.
The results of the present study show that CMA3 staining can be carried out, by FCM, in the presence or absence of fixative. Comparison of the same samples fixed with carnoy's solution and unfixed show that fixation reduces CMA3 positivity in FCM (Fig 2). On possible hypothesis for this difference is the lower ability of CMA3 to enter the chromatin in a fixed sample and attach to unprotaminated DNA. Indeed treatment of samples with DTT, a disulfide reducing agent which helps to remove protamine and exposes the DNA to CMA3, significantly increases CMA3 positivity (Fig 1).
Considering the mean value of unfixed FCM and fluorescence microscopy were similar (not statistically different); however in some samples the CMA3 values assessed by fluorescence microscopy, were not in accordance with the FCM results especially when assessed unfixed. Therefore, we assessed the coefficient of correlations between these three procedures. The results revealed a strong significant positive correlation between fixed with unfixed in FCM and a weak significant correlation between fixed samples by FCM with fluorescence microscopy, while no significant correlation was obtained between unfixed samples with FCM and fluorescence microscopy. These results may account for lower credibility of the microscopic procedure. This difference may be accounted by instrumental precision and more uniformity of staining in the FCM (in the tube rather than slids) in addition to variations such as inter and intra assay variation.
The effect of sperm number exposed to fixed volume of CMA3 was also assessed. Unlike the fluorescence microscope procedure, the results show a significant decrease in percentages of CMA3 positivity with increased number of sperm exposed to fixed volume of CMA3 in FCM (Fig 3A) , thus suggesting that a fixed number of spermatozoa should be used during CMA3 assessment in order to compare results within or between experiments. The reason for this observation is that with increase number of sperm, higher CMA3 binding sites are available and therefore, the number of sperm to fixed volume of CMA3 must be maintained during CMA3 assessment. Following this observation, we evaluated effect number of sperm on percentages of CMA3 positivity in the fluorescence microscope procedure. The result showed that the number of spermatozoa fixed on each slide did not affect the results of CMA3staining (Fig 3B).
In order to evaluate the lowest number of sperm required for assessment of CMA3 positivity by FCM, both number of sperm and the volume of CMA3 solution were reduced proportionally to maintain the final concentration of CMA3. The results revealed that the lowest sperm number required for FCM was 1 million sperm (Fig 3C) to assess CMA3 value in oligozoospermic samples. Lower sperm concentration may affect the validity of results.
The other aim of this study was optimization of duration of exposure to CMA3. The results show that, when samples were fixed, the percentage of CMA3 positivity increased gradually but reached a steady state between 40-60 minutes and subsequently increased, while in unfixed samples the percentage of CMA3 positivity increased gradually with time (Fig 4). Although it is difficult to explain the difference observed between fixed and unfixed sample, but one possible explanation may be due to the fact that in the fixed sample protamine cannot be easily displaced and chromatin saturation by CMA3 is reached with a time

point, while in the unfixed samples breakage of disulfide bridges may take place due to auto-oxidation. Therefore, one may propose to fixed samples and expose them to CMA3 for 6o minutes so that CMA3 reaches a steady state and the results are not affected by variation in exposure time. Conversely, samples could be assessed unfixed but it is important to note small variations in time may affect CMA3 positivity in FCM.

Conclusion

Considering the value of CMA3 assessment in the management of infertility and research and the use of FCM in two previous studies based on the slide method, we propose FCM is a suitable, precise and accurate method for assessment of CMA3 staining, however it should be standardize. Therefore, we advise researchers to consider the following points during CMA3 assessment by FCM: 1) use fixed samples, 2) use fixed number of sperm per ml (2 million), 3) expose samples for 60 minutes to CMA3 solution and 4) samples can be fixed, stained, washed and assessed later. The factors which affect FCM and may not affect the slide method are: sperm concentration and duration of exposure to CMA3.

Acknowledgements

The authors express their gratitude to the Royan Institute for its financial support, as well as the staff of Isfahan Fertility and Infertility Center. There is no conflict of interest in this study.

References

1. Hargreave T. Genetically determined male infertility and assisted reproduction techniques. J Endocrinol Invest. 2000; 23: 697-710.
2. Cordelli E, Eleuteri P, Leter G, Rescia M, Spanò M. Flow cytometry applications in the evaluation of sperm quality: semen analysis, sperm function and DNA integrity. Contraception. 2005; 72: 273-279.
3. Mortimer D. The future of male infertility management and assisted reproduction technology. Hum Reprod. 2000; 15: 98-110.
4. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, et al. Utility of sperm chromatin structure assay (SCSA) as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod. 1999; 14: 1039-1049.
5. Amann RP. Can the fertility potential of a seminal sample be predicted accurately? J Androl.1989; 10: 89-98.
6. Evenson DP, Parks JE, Kaproth MT, Jost LK. Rapid determination of sperm cell concentration in bovine semen by flow cytometry. J Dairy Sci.1993; 76: 86-94.
7. Thomas CA, Garner DL, DeJarnette JM, Marshall CE. Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. Biol Reprod. 1997; 56: 991-998.
8. Levek-Motola N, Soffer Y, Shochat L, Raziel A, Lewin L.M, Golan R. Flow cytometry of human semen: a preliminary study of a noninvasive method for the detection of spermatogenetic defects. Hum Reprod. 2005; 20: 3469-3475.
9. Johnson LA, Flook J, Look M, Pinkel D. Flow sorting of X and Y chromosome-bearing spermatozoa into two populations. Gamete Research.1987; 16: 1-9.
10. Johnson LA, Flook JP, Hawk HW. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod. 1989; 41:199-203.
11. Perreault SD, Aitken RJ, Baker HW, Evenson DP, Huszar G, Irvine DS, et al. Integrating new tests of sperm genetic integrity into semen analysis: breakout group discussion. Adv Exp Med Biol. 2003; 518: 253-268.
12. Angelopoulou R, Plastira K, Msaouel P. Spermatozoal sensitive biomarkers to defective protaminosis and fragmented DNA. Reprod Biol Endocrinol. 2007; 5: 36.
13. Nasr-Esfahani MH, Razavi S, Mardani M. Relation between different human sperm nuclear maturity tests and in vitro fertilization. J Assist Reprod Genet. 2001; 18: 219-225.
14. Saowaros W, Panyim S. The formation of disulfide bonds in human protamines during sperm maturation. Experientia. 1979; 35: 191-192.
15. Nasr-Esfahani MH, Aboutorabi R, Razavi S. Credibility of Chromomycin A3 Staining in Prediction of Fertility. International Journal of Fertility and Sterility (IJFS). 2009; 9: 5-10.
16. Nasr-Esfahani MH, Salehi M, Razavi S, Anjomshoa M, Rozbahani S, Moulavi F, et al. Effect of sperm DNA damage and sperm CMA3 staining on fertilization and embryo development post-ICSI. Reprod Biomed Online. 2005; 11: 198-205.
17. Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas D. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. Biol Reprod. 1993; 49: 1083-1088.
18. Razavi S, Nasr-Esfahani MH, Mardani M, Mafi A, Moghdam A. Effect of human sperm chromatin anomalies on fertilization outcome post-ICSI. Andrologia. 2003; 35: 238-243
19. Bianchi SD, Lojacono A. The role of the traditional radiological methods in conservative therapy and endodontics. Minerva Stomatol. 1996; 45: 575-587.
20. Iranpour FG, Nasr-Esfahani MH, Valojerdi MR, al-Taraihi TM. Chromomycin A3 staining as a useful tool for evaluation of male fertility. J Assist Reprod Genet. 2000; 17: 60-66.
21. Nasr-Esfahani MH, Razavi S, Tavalaee M. Failed fertilization after ICSI and spermiogenic defects. Fertil Steril. 2008 ;89: 892-898.
22. Tavalaee M, Razavi S, Nasr-Esfahani MH. Effects of spermiogenesis defects on fertilization and pregnancy rate in IVF patients. Yakhteh. 2007; 9: 103-110.
23. Tarozzi N, Nadalini M, Stronati A, Bizzaro D, Dal Prato L, Coticchio G, et al. Anomalies in sperm chromatin packaging: implications for assisted reproduction techniques. Reprod Biomed Online. 2009; 18: 486-495.
24. Simões R, Feitosa WB, Mendes CM, Marques MG, Nicacio AC, de Barros FR, et al. Use of chromomycin A3 staining in bovine sperm cells for detection of protamine

deficiency. Biotech Histochem. 2009; 84: 79-83.
25. World Health Organization. Laboratory manual for the examination of human semen and semen-cervical mucus interaction. Cambridge, UK: Cambridge University Press, 1999.
26. Oliva R. Protamines and male infertility. Hum Reprod Update. 2006; 12: 417-435.
27. Braun RE. Packaging paternal chromosomes with protamine. Nat Genet. 2001; 28: 10-12.
28. Carrell DT, Emery BR, Hammoud S. Altered protamine expression and diminished spermatogenesis: what is the link? Hum Reprod Update. 2007; 13: 313-327.
29. Aoki VW, Liu L, Jones KP, Hatasaka HH, Gibson M, Peterson CM, et al. Sperm protamine 1/protamine 2 ratios are related to in vitro fertilization pregnancy rates and predictive of fertilization ability. Fertil Steril. 2006; 86: 1408-1415.
30. Nasr-Esfahani MH, Salehi M, Razavi S, Mardani M, Bahramian H, Steger K, et al. Effect of protamine-2 deficiency on ICSI outcome. Reprod Biomed Online. 2004; 9: 652-658.
31. Gledhill BL, Evenson DP, Pinkel D. Flow cytometry and sorting of sperm and male germ cells. In: Melamed MR, Lindmo T, Mendelsohn ML (editors). Flow cytometry and sorting. 2nd ed. New York: Wiley-Liss; 1990; 531- 551.