Research in Molecular Medicine

Original Research Article

2016, Vol: 4, Issue: 1, Pages: 50-55

DOI: 10.7508/rmm.2016.01.008

Evaluation of Protamine Level in Human Sperm Samples Using Chromomycin A3 and Aniline Blue Staining

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Received: 4 Nov 2015

Revised : 13 Dec 2015

Accepted: 3 Jan 2016

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Abstract

Background: Current microscopic experimental methods cannot diagnose DNA damages present in spermatozoa. Therefore, some methods are needed to address the abnormality of the genetic material status on the sperm samples. As reported by many investigators aniline blue staining technique has been used for identifying sperm chromatin condensation. Also, chromomycin A3 is used for evaluation of the degree of protamination of spermatozoa. This study aimed at evaluating these two different staining techniques on human sperm protamine status.

Materials and Methods: Sperm samples were collected from 72 males [including 37 infertile men: (seven asetenotratospermic, two trato-espermic, and one azo-spermic) and 35 healthy fertile men] attending the research and clinical center for infertility affiliated with Babol University of Medical Sciences. Measurement of sperm motility, volume and density of semen samples were carried out in andrology laboratory. In estimation with light microscopy aniline blue tool, in each slide, blue stained were assumed as normal spermatozoa, but dark blue stained were regarded as abnormal spermatozoa. Bright yellow stained chromomycin-reacted spermatozoa (CMA3+) were observed under fluorescent microscope with 460 nm filter considered as normal and yellowish green were assumed as abnormal. Statistical analysis results were expressed as mean ± SD.

Results: The rate of reacted spermatozoa to aniline blue in the infertile group was higher than that of the healthy control group 42.8% ±8.7 vs. 17.9% ±6.4. Also, the rate of reacted spermatozoa to CMA3 in infertile and normal group was [53.6 ± 8.7 and 24.7% ±5.1], respectively.

Conclusion: Infertility status could be assessed by staining the spermatozoa via aniline blue and CMA3 techniques. Combination of these two staining methods had the best predictive values for semen analysis compared to using just one method. Our results showed that both CMA3 and AB staining methods were successful in detecting sperm chromatin defects.

Keywords: Aniline Blue; Chromomycin A3; Chromatin condensation; Chromatin structure; Protamine deficiency

Please cite this article as: Qujeq D, Evaluation of Protamine Level in Human Sperm Samples Using Chromomycin A3 and Aniline Blue Staining. Res Mol Med. 2016; 4 (1): 50-55

Introduction

Assessing sperm chromatin condensation was used by chromomycin A3 and aniline blue techniques to examine the integrity of the DNA in sperm. Literature review confirmed that abnormal sperm chromatin condensation was not correlated withfertilization rate (1). Many reports indicated that the standard semen analysis and many surface operation methods were used to improve sperm condition and sperm stability (2). Protamine defect, were estimated by chromomycin A3 staining technique, do address the integrity of the genetic material of the sperm (3). Chromomycin

A3 positivity is a demonstration of protamine defect could be a cause of men infertility. Chromomycin A3 staining technique could be considered as a useful test for evaluation of male fertility prior to infertility treatment (4). Semen analysis, involving chromomycin A3 and aniline blue staining was performed in an infertile people to establish to evaluate mechanism of infertility. Many studies showed a correlation between sperm chromatin defects and reduced ability of fertility (5). The sperm chromatin character, was evaluated with many tests such as: aniline blue, toluidine blue, acridine orange and chromomycin A3 staining techniques (6). Application of the human sperm chromatin experience physiological remodeling during in vitro capacitation and acrosome reaction is well accepted. This method is used to detect the presence of injury in sperm (7). Acidic aniline blue staining for excessive histones and chromomycin A3, for protamine deficiency tests represent a valuable baseline information for the degree of chromatin condensation (8). Chromomycin A3 staining a deoxyribonucleaic acid specific fluorescent probe, and aniline blue staining techniques were used to evaluate the effect of sperm chromatin anomalies on fertilization (9). As reported by many researchers the most important and applicable methods of studying sperm chromatin status include aniline blue, which shows the presence of excessive histones (10) and chromomycin A3 (CMA3) which demonstrates protamine deficiency (11). CMA3 is a flurochrome which has been shown to compete with the protamines for binding to the minor groove of DNA and detecting protamine deficiency in loosely packed chromatin and is correlated with extent of nicked DNA (12). chromomycin A3 (CMA3), a fluorochrome specific for GC-rich sequence, is used for evaluation of the degree of protamination of mature spermatozoa (13). During spermatogenesis, DNA histones are mainly replaced by protamines in order to acquire a highly packed sperm chromatin structure. Another indirect approach is the use of aniline blue staining to detect the presence of extra histones and, therefore, indirectly infer the presence of lower amounts of protamines in the sperm nucleus. In other words, aniline blue staining detects presence of remaining histones by binding to lysine-rich histones (14). Aforementioned techniques are currently employed for evaluating the nuclear chromatin integrity of sperms. These include aniline blue staining, which selectively stains lysine-rich histones (15). CMA3 and AB staining methods are regarded as valuable methods for evaluating the sperm's chromatin quality (16). Between staining methods on human sperm protamine, CMA3 is the sensitive and specific test for in vitro fertilization (17). The aim of this study was to assess the amount of semen protamine based on staining and fluorochromes methods.

Materials and Methods

Sperm samples

Samples were collected from infertility and reproductive health center in Babol University of Medical Sciences, babol, Iran. To study sperm chromatin status, the sperm samples were collected from 72 males including 37 infertile men: seven asetenotratospermic, two trato- espermic, and one azoo-spermic, and 35 healthy fertile men). Measurement of motility, volume and density of semen samples were carried out in andrology laboratory. The patients were classified into two groups: infertile men (n = 32) and healthy control (n = 35). Inclusion criteria were as follows: Ssubjects who had no child after a period of unprotected intercourse for more than 2 years with various abnormal semen parameters according to the recommendations of the World Health Organization (1999) were considered as infertile. Also, healthy control men with proven fertility who had a successful fertility within the last two years and normal spermogram at the time of study were selected as control and fertile group. Exclusion criteria were as follows: Ttreatment with drugs known affecting to the sperm quality and sever diseases. Also, azoospermic samples were excluded from the study. The study protocol was approved by our University research ethics committee (no=39-65).

Semen analysis

All specimens were collected by expert technician and delivered to the laboratory. Measurements of motility, volume and density of each semen sample were carried out in andrology laboratory. Also, routine semen analysis was performed according to WHO criteria (World Health Organization, 1999) and staining was applied for sperm morphology evaluation.

Aniline blue staining

Aniline blue staining method was also performed as previously described (18), aniline blue, water soluble (sigma) was prepared as a 2.9% (w/v) solution in phosphate- buffer saline (PBS), pH 7.0 and diluted as indicated for staining fixed cells. Cells in suspension were fixed in 4.5% paraformaldehyde and stained with an equal volume of a 0.45% dilution of aniline blue. Air-dried smears were prepared from fresh semen samples of each study all participants, fixed in 5% buffered glutaraldehyde in 0.45 m phosphate buffer (pH 7.0) for 25 min at room temperature. Each smear was stained with 2.9% aqueous AB stain in 5.5% acetic acid (pH 3.5) for 12 min.

In light microscopic evaluation (Olympus, Tokyo,

Japan), all spermatozoa were counted in each slide and unstained or pale blue stained were considered as normal spermatozoa while dark blue stained wereregarded as abnormal spermatozoa.

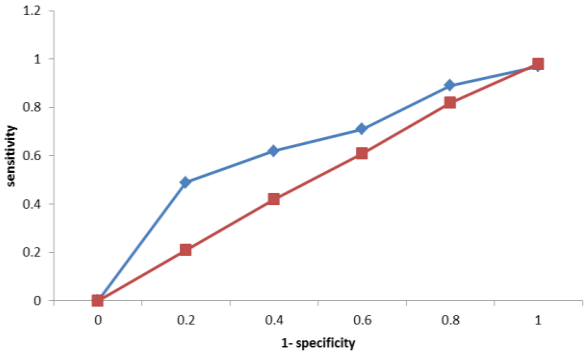

Figure 1. ROC curves for aniline blue method. Each point represents the mean ± SD of 6 experiments.

Chromomycin A3 staining

Chromomycin A3 staining method was performed as previously described (18). Smears were first dried and then fixed in solution including (methanol/glacial acetic acid, 3: 1) at 50C for 12 min. Each slide was treated with 120 µl of CMA3 (0.35 mg ml) in buffer; 5 ml citric acid, 0.15 mM and 37 ml Na2HPO4,7H2O, 0.45 mM, pH 7.0 containing 8.2 mM MgCl2) for 24 min. After staining, the slides were washed in buffer and immobilized with buffered glycerol (1: 1). Bright yellow stained chromomycin-reacted spermatozoa (CMA3+) and yellowish green stained non-reacted spermatozoa (CMA3) were viewed under fluorescent microscope with a 460-nm filter (Labor lux 12, Germany).

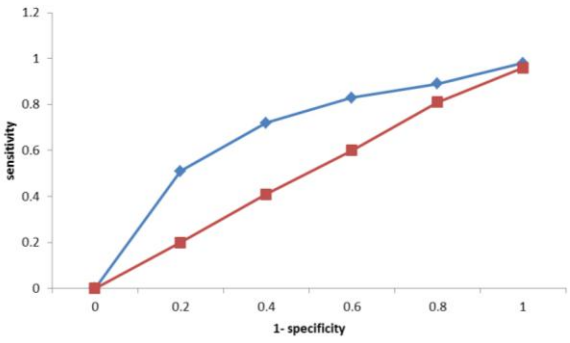

Figure 2. ROC curve for CMA3 method. Each point represents the mean ± SD of 6 experiments.

Statistical analysis

Results are expressed as the mean ± SD. Statistical data analyses were assessed using students t-test with $P<0.05$ as the minimal level of significance.

Ethics Statment

Research protocol was approved by the Medical Ethics Committee of Babol University of Medical Sciences (no: 39-65).

Results

Chromatin packaging quality was estimated by staining with chromomycin A3 and aniline blue techniques, which marked spermatozoa with defective packaging.

The sperm nuclear integrity in the two groups of infertile and healthy control men are shown in table 1.

Table 1. The rate of reacted spermatozoa to aniline blue, chromomycin A3 staining.

Staining method	Fertile group	Infertile group
AB+ (%)	17.9 ± 6.4	42.8 ± 8.7
CMA3+ (%)	24.7 ± 5.1	53.6 ± 7.5

Each assayed in duplicate, $P<0.05$.

The rate of reacted spermatozoa to aniline blue in the normal and infertile group was [17.9 % ±6.4 and 42.8 % ±8.7], respectively. The difference between the rates of AB-reacted spermatozoa (AB+) in infertile and control groups was significant ($P<0.05$). The rates of reacted spermatozoa to CMA3 in the normal and infertile groups waswere [24.7 % ±5.1 and 53.6 ± 8.7], respectively.

The predictive values of TTV, and the rates of aniline blue reacted spermatozoa (AB+) for discriminating between fertility and infertility were assessed by calculating the corresponding area under ROC curve (AUC), (Figure 1).

On a standard alone basis, the rates of aniline blue reacted spermatozoa (cut off at 42.8 %, AUC 0.71) was the best predictor of the rates of aniline blue reacted spermatozoa outcome, with a PPV and NPV of 78.4 and 69.5, respectively and with LR+ and LR- of 1.96 and 0.68, respectively.

The predictive values of TTV, the rates of chromomycin A3 reacted spermatozoa (CMA3+) for discriminating between fertility and infertility were assessed by calculating the corresponding area under ROC curve (AUC), (Figure 2).

On a standard –alone basis, the rates of chromomycin A3 reacted spermatozoa (cut off at 53.6 %, AUC 0.82) was the best predictor of the rates of chromomycin A3 reacted spermatozoa outcome, with a PPV and NPV of 83.2 and 71.3, respectively and with LR+ and LR- of 1.98 and 0.74, respectively.

After multivariate logistic regression analysis, TTV aniline blue and chromomycin A3 were retained as being independently correlated with fertility outcome.

The PPV, NPV, LR+ and LR – were also evaluated for various ratio [aniline blue/ chromomycin A3; aniline /TTV (aniline×TTV)/ chromomycin A3; aniline blue / (TTV× chromomycin A3)] were also evaluated.

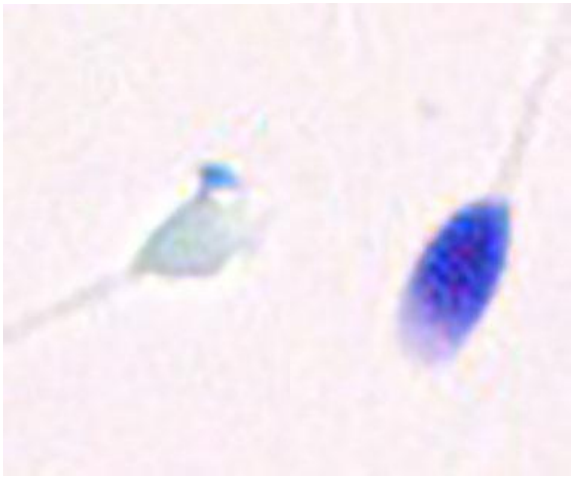

Figure 3. Aniline blue staining. Un-stained or pale blue stained, non-reacted spermatozoa were considered as sperm with normal protamine (left). Dark blue stained, reacted spermatozoa were considered as sperm with low protamine (right). Stained spermatozoa were evaluated with light microscopy.

As Figure 3 shows aniline blue bind to histones. CMA3 substance molecule is a fluorochrome specific to bind the DNA compared to protamine, as shown in Figure 4.

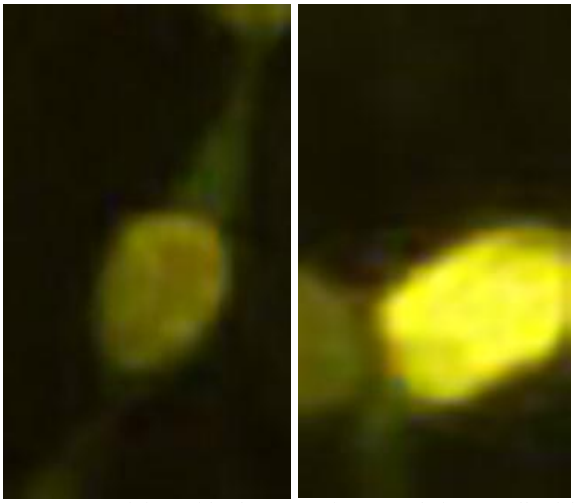

Figure 4. Staining with chromomycin A3 technique. Yellowish green stained non-reacted spermatozoa were considered as sperm with normal protamine (left) . Bright yellow stained reacted spermatozoa were considered as sperm with low protamine and fluorescence radiation was shown (right).

Discussion

In infertile men, there was a higher percentage of abnormal spermatozoa after staining with aniline blue and chromomycin A3. The results showed a positive relationship between both mentioned staining techniques and the analysis of the various sperm characteristics. Our results also indicated that abnormal sperm characteristics are correlated with protamine's deficiency detected by both AB and CMA3 staining techniques. The present study found the chromatin compaction status in correlation with fertility and non-fertility status.

According to the present study, infertility status can be investigated with the aid of aniline blue staining and chromomycin A3 tests. In addition, these tests provided valuable baseline information about human sperm chromatin.

The main findings of the present study were that the rate of reacted spermatozoa to aniline blue in the infertile group was higher than those of the healthy fertile group, which shows the presence of excessive histones in infertile men. Also, the rate of reacted spermatozoa to chromomycin A3 in the infertile was higher compared to that of the healthy fertile group, which demonstrates protaimine level deficiency in infertile men. Therefore, evaluation of the sperm's quality via combination of CMA3 and AB staining technique could be considered for human sperm analysis. Sperm abnormal chromosomal condensation induced by protamine deficiency may be considered as an alternative cause of failed fertilization.

The sperm chromatin condensation was shown with application of two assays of AB and CMA3 techniques. Aniline blue is a marker for detection of extra lysine-rich histone proteins, while CMA3 detects protamine defects during histone– protamine replacement in the testicular phase of sperm chromatin condensation. The rates of AB-reacted spermatozoa (AB+) was different in infertile and normal groups. In present study some interesting similarities were found between current findings and those reported earlier (16). Our results were in good agreement with those reported previously, (16) that observed a higher percentage of stained spermatozoa after staining with AB in infertile men than in healthy control men. In many studies, the chromatin of spermatozoa in relation with fertility status has been assessed by staining the spermatozoa with aniline blue and CMA3. A correlation has been reported to exist between abnormal sperm chromatin as evaluated by CMA3 staining and the presence of DNA damage. These results confirm findings of other investigators (17, 18) who used CMA3 for detecting protamine deficiency in infertile men.

The results of ROC curves analysis showed that aniline blue staining method was more predictable than CMA3 staining techniques. The difference in results may come from different kinds of samples.

Also, other factors such as age and time point of

infertility may have been due to the apparent discrepancy between current findings and those of theother results (17, 19). The multivariate logistic regression analysis had the best predictive values for fertility outcome (AUC: 0.83). Estimation of sperm's characteristic via combination of CMA3 and AB staining methods could be assumed as one of the perfect methods in human sperm analysis. These methods could address the integrity of the genetic material of sperm. Present results revealed that higher numbers of AB and CMA3 positive in infertile men compared to fertile men.

Conclusion

Our results demonstrated a difference in percentage of AB and CMA3 reacted spermatozoa between fertile and in-fertile groups. Sperm chromatin integrity analysis by AB and CMA3 staining methods demonstrate a significant decrease in protamine in non-fertile men compared to fertile men. The percentage of CMA3 positive spermatozoa had a significant positive correlation with the percentage of spermatozoa stained with aniline blue staining and sperm characteristics. AB and CMA3 staining methods can detect the sperm abnormality, so they are considered as important means for detection of sperm chromatin injury.

Acknowledgements

The authors would like to express thanks to Dr Ali Asgher Sephidger for his help in allowing the use of laboratory equipment (fluorescent microscope) and Dr Gholamali Joursaraie for his advice and help in sampling and Miss Asefeh Haghghi for her help in sampling. Also, my appreciation goes to the staff in Infertility and reproductive health research center, Health Research Institute of Babol University of Medical Sciences, Iran for their help in sampling.

Author contributions

QD Performed experiments and wrote the manuscript.

Conflict of interest

No competing financial interests exist.

Support/Funding

This work was supported by grant (no=923739) from the research council of Babol University of Medical Sciences.

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