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DNA FRAGMENTATION INDEX (DFI) OF HUMAN SEMEN BY MODIFIED ANILINE BLUE METHOD

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ABSTRACT

Sperm chromatin condensation forms an important criterion in the assessment of functional potential of the spermatozoa. It is gaining more importance in the scenario of rising infertility rates and advanced infertility management modalities like IVF and ICSI. During the condensation of sperm nucleus histones in DNA are replaced by protamines. Acidic Aniline Blue stain evaluates sperm chromatin defects by differential staining of lysine rich histones and arginine and cysteine rich protamines in the sperm nuclei. It has an advantage that the slides can be examined under light as well as fluorescence microscope. The current study is conducted with the aim of studying the DNA Fragmentation Index (DFI) based on chromatin compaction by using Acidic Aniline Blue stain. The impact of nuclear integrity and DFI on assisted reproduction techniques and outcome is discussed in the paper.

Key Words: *Sperm Chromatin Condensation, Acidic Aniline Blue Stain, DNA Fragmentation Index (DFI), Infertility*

INTRODUCTION

Sperm DNA integrity is essential for accurate transmission of genetic material to the offspring and birth of a healthy child. Since rate of infertility is increasing, being about 10 to 15 %, many assisted reproduction techniques [ART] like In-vitro fertilization [IVF] and intra-cytoplasmic sperm injection [ICSI] are widely used.

The success of ART with normal embryogenesis and foetal development depends on many different semen parameters like total sperm count, motility, morphology and vitality. The sperm chromatin compaction is another parameter which is less frequently assayed but may lead to repeated ART failures. The compaction occurs by a complex mechanism in which initially the histones are replaced by transition proteins and finally by protamines (Oliva, 2006). This maturity of sperm head is essential for prediction of the fertilizing capacity of sperms. The addition of evaluation of sperm head maturity to routine semen analysis improves the assessment and choice of infertility treatment. There are many analytical techniques for assessment of human sperm chromatin integrity.

Assays like Acidic Aniline Blue staining, Toluidine Blue staining, Chromomycin A3 assay, Fluorescent in situ hybridization assay, COMET and TUNEL (Agarwal, 2004) are used for sperm chromatin evaluation. It is observed that though there is a great clinical relevance these assays are rarely done during semen evaluation for infertility. It may be partly because of its high cost and partly due to lack of infrastructure, instruments and trained personnel. Aniline blue staining evaluates the degree of Sperm Chromatin Compaction and sperm chromatin defects on the basis of its nucleoprotein content. The acidic aniline blue binds to the lysine rich histones in the sperm head. It stains their heads dark blue as compared to normal, mature DNA which has arginine and cysteine rich protamines which do not bind to aniline blue. Hence mature sperm heads appear unstained in the smears.

Aniline blue stain also has an advantage of providing slides which can be seen under bright field microscope. However a proper evaluation is sometimes difficult because the mature sperms remain unstained. In this study we propose a modification of Acid Aniline Blue staining method. A counter stain eosin and safranin when used after aniline blue enhanced the staining. This made the identification and differentiation of mature condensed sperm heads from immature heads easier.

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MATERIALS AND METHODS

After informed consent and ethical committee approval all Semen samples coming for infertility assessment to the laboratory were collected in the period of 4 months. Samples were assayed for routine parameters and divided into three aliquots – A, B, C. Slides were prepared by feathering method as described in the WHO manual (WHO Manual for semen analysis, 2010).

A. Acidic aniline blue staining AAB

Slides were air dried, fixed in 3% buffered Glutaraldehyde for 30 minutes and stained with AAB for 5 min (Agarwal, 2004; Sellami *et al.*, 2013). Sperm heads were observed under oil emersion and 200 sperms counted. The percentage of darkly stained sperms was the DNA fragmentation index.

B. AAB counterstained with eosin AAB-E

The AAB slides were further dipped in aqueous 0.5% Eosin for 3 min. The immature sperm heads remained dark blue while mature sperm heads stained red-pink

C. AAB counterstained with safranin

The AAB slides were stained with 0.5% Safranin by two dips, air dried and observed under oil emersion. Immature heads stained blue-purple. Mature sperms stained dark pink.

RESULTS AND DISCUSSION

Observation and Results

The sperm heads were differentiated under oil immersion into normal and abnormal with fragmented DNA on replicate smears, counting 200 spermatozoa and by two different observers to reduce error. The differentiation was made easier due to contrast staining in methods B and C.

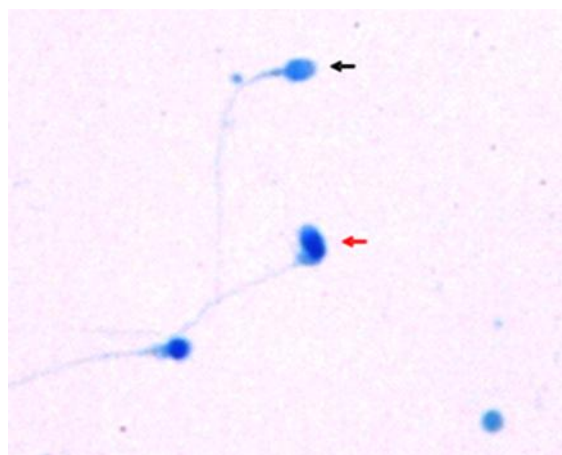
1] Out of twenty eight samples examined for semen analysis in the given period of time eight were azoospermic and were not stained further.

2] Out of twenty samples, sixteen semen samples [80 %] had DFI> 20 % which is indicative of high DFI. All these patients were undergoing infertility treatment.

3] Our readings correlated well by using AAB as well as its modifications but AAB-S gave more clarity and less chance of interobserver variations.

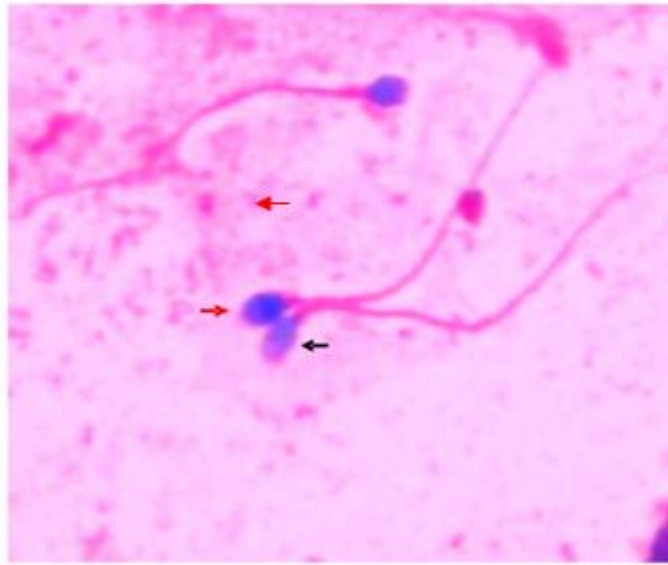
4] The statistical analysis of the data was done using SPSS software and Pearson correlation coefficient. AAB method was compared with AAB-E. The Pearson correlation coefficient was 0.941 with $p < 0.05$, thus showing statistically significant correlation.

AAB method was compared with AAB-S. The Pearson correlation coefficient was 0.94 with $p < 0.05$, thus showing statistically significant correlation

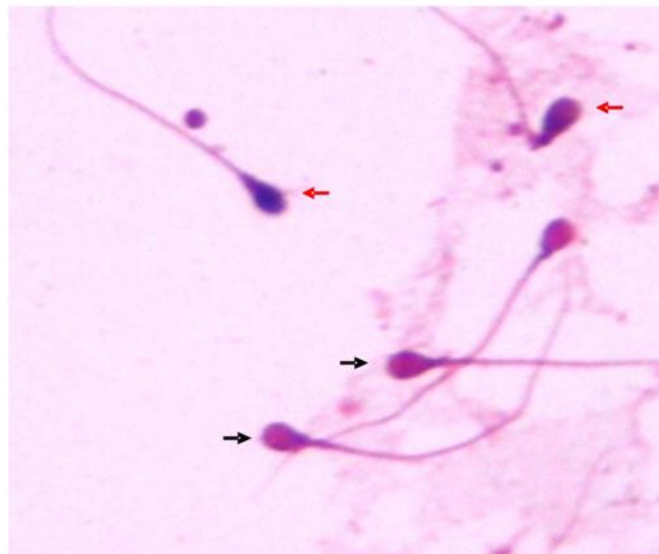


A. Acidic Aniline Blue (AAB)

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B. Acidic Aniline Blue with Eosin (AAB - E)



C. Acidic Aniline Blue with Safranin (AAB - S)

Figures A, B, C: Oil Immersion (1000 X) photographs showing acidic aniline blue, acidic aniline blue with eosin and acidic aniline blue with safranin respectively. The red arrows (↔) indicate darkly stained sperm heads with abnormal chromatin condensation and black (↔) arrows indicate faintly stained normal sperm heads

Discussion

Sperm chromatin compaction or condensation plays a vital role in transmission of normal genetic material to the zygote. It has an impact on outcome of ART and many failures of ART, recurrent abortion minor or major congenital anomalies may be related to high DFI of sperms used for IVF or ICSI (Esfahan, 2001; Mohammad, 2012; Lin, 2008; Sakkas, 1998). Our study revealed 80% semen samples with DFI>

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20. Review of literature indicates that a DFI of > 20 % suggests a higher rate of ART failures (Sellami 2013). All these patients were under infertility treatment and such a high DFI would certainly play a significant role in their further management, ART options and outcome. Increased histone remnants in the sperm head leads to premature chromatin condensation and this may be the cause of failure in fertilization and / or embryo development (Sakkas, 1998). The inclusion of sperm chromatin condensation and DFI would be a valuable parameter in a semen analysis of such patients of male infertility before any ART procedure.

Yet there are some contrasting reports on effect of sperm nuclear damage and fertilization rates (Hammadah, 1999). Hence there is a need for obtaining more data on DFI and its clinical correlation. There are several sophisticated and expensive methods for DNA fragmentation assays. Moreover it is of great importance for developing countries to develop and standardize simpler and cost effective techniques which could be utilized by all peripheral laboratories for reporting DFI in semen analysis.

Our AAB staining method with some modification using Eosin and Safranin which showed statistically significant correlation between all three methods provide best and easy interpretation of slides. Further it is cost effective and would not burden the couples undergoing ART who are already socially, psychologically and economically disturbed. In this paper we highlight the need for evaluation of DFI in all couples undergoing ART for infertility management. Further studies are needed to gain more data which is essential for correlating these parameters and pregnancy outcome in such couples.

ACKNOWLEDGEMENT

I express my gratitude to the V.C, Dean, Dr. Patki and my colleagues for encouragement and expert guidance during this project.

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