Original Article

The effects of protamine deficiency on ultrastructure of human sperm nucleus

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Abstract

Background: Chromomycin A3 (CMA3) staining is one of the staining methods for detecting protamine deficiency in sperm nucleus. CMA3 is a fluorochrome that competes with protamines for binding to DNA double helix. It has been shown in our previous studies that percentage of CMA3 positive spermatozoa in semen has a close significant relationship with the fertilization rate in *in vitro* fertilization (IVF). The aim of this study was to examine the ultrastructural differences between sperms in patients who had high fluorescent percentages of yellow or red in CMA3 staining (protamine deficient) with patients with low fluorescent percentages.

Materials and Methods: Semen samples are taken from five patients with high fluorescent percentages and five patients with low fluorescent percentages. Then the samples are passed for the different steps of preparing for electron microscopy. After the sectioning and mounting on grids, they are investigated under the transmission electron microscope.

Results: Sperms in patients with low percentages of positive spermatozoa often have a normal appearance. Sperms in high fluorescent samples frequently have unpacked chromatin. Furthermore acrosomes of these sperms are thinner or disturbed. Also sometimes there are irregularities in sperm head membrane.

Conclusion: Protamine deficiency in sperm nucleus can cause ultrastructural anomalies in sperm chromatin such as unpacking of it. It also is concomitant with acrosome and sperm membrane disturbances.

Key Words: Chromatin, chromomycinA3, spermatozoa, ultrastructure

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INTRODUCTION

Human sperm is one of the first cells, which was observed under microscope by Lee Van Hook. Electron microscope is a device used for examining the ultrastructure of cells. Today, transmission electron microscopy is used in many studies to examine ultrastructure of sperms in patients with chromosomal defects such as translocation of chromosomes 10 and 15,[1] microdeletion of chromosome Y,[2] Robertsonian translocation,[3] and aneuploidy of chromosomes Y, X, and 18.[4,5] Besides, this microscope is applied to examine DNA fragmentation in sperms before and after applying Swim Up technique. It has been shown that preparing sperms by Swim Up method reduces necrotic or apoptotic sperms.[6] In addition, microdeletion of chromosome Y in patients with

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oligospermia was investigated by Mantas *et al*.[7] Another study was conducted on nuclear abnormalities in sperms with long heads.[8] Some studies have been also conducted on fertile and infertile patients and the effects of smoking on fertility and ultrastructure of sperm nucleus.[9-11]

Human sperm chromatin becomes very stable in physical terms and inactive in genetic terms during spermiogenesis.[12] Such a change is caused by DNA complex with special nuclear proteins called protamine.[12,13] In mammalians, these protamines contain a large amount of amino acids, arginine and cysteine, and so many disulfide intermolecular and intramolecular bridges are observed in them.[14] Sulfhydryl bonds are oxidized while passing through the epididymis and provide great stability to sperm nucleus.[14] As a result of this stability, nucleus of sperms in mammalians is extraordinarily resistant to destruction by mechanical factors (like sonication).[14]

Under electron microscope, sperm nucleus seems as a high condensation mass. Natural sperm nucleus has carotenoid-like appearance and its structure is strongly formed by mixed DNA and blended with alkaline proteins. Structure, components and appearance of a mature sperm are produced by deep chemical and physical changes, which start in late stages of spermiogenesis inside seminiferous tubules of testes and are completed in epididymis.[15] These changes include RNA removal, replacing somatic histones with protamines, and forming disulfide bonds for chromatin stabilization. Structural changes of sperm along with acrosome growth and gradual condensation of chromatin lead to lengthening of sperm nucleus.[15] Gradual condensation of chromatin makes sperm chromatin highly condensed at the end. As a result of these changes, mature sperm nucleus will be strongly resistant to the probable chemically and physically generated lesions. According to many investigators, this degree of inactivity is required for protecting male genome against physical, chemical, and mutagenic factors.[16] Only within cytoplasm of egg cell and under special physiological conditions, disulfide bonds are split and histones with origin of egg cell replace protamines; thus, sperm becomes uncondensed.[17]

In general, structural abnormalities in sperm nucleus include two kinds of incomplete condensation of nucleus and the presence of nuclear vacuoles. Nucleus of the sperms with insufficient condensation has coarse grains under electron microscope, which is the special characteristic of late spermatids. Thus, this phenomenon is sometimes called immaturity of chromatin.[17,18] This lesion occurs either by itself or together with acrosome lesions. Medium condensation of chromatin by itself limits fertility.[19] The presence of nuclear lacunae or vacuoles is another abnormality in sperm nucleus found through studying ultrastructures. Nuclear lacunae are in fact hollow zones in sperm nucleus. In fact, such holes are rarely observed even in sperms of normal people and their presence indicates that gradual condensation of chromatin in late spermatid stage does not always occur with full condensation of nucleus size. In the case of this lesion, all or some of the sperms in semen have large and often multiple lacunae, which significantly reduce chromatin mass and greatly transform the shape of nucleus. This change in nucleus shape affects the spatial relationship of nucleus with acrosome.

Chromomycin A3 (CMA3) is a fluorochrome, which is able to bind to minor groove of double helix[1]. It seems that CMA3 competes with protamines to access DNA.[20] So, protamine deficiency in chromatin of sperm nucleus leads to staining of sperm nucleus chromatin showing yellow or red fluorescence under the microscope.[21] Therefore, the amount of staining with CMA3 is in direct relationship with the amount of protamines used in nucleus. Accordingly, nuclei of sperms with protamine deficiency are seen green with CMA3 staining, and those with sufficient protamine are seen fluorescent yellow or red.[21]

The previous study of the present authors demonstrated that high fluorescence in the samples collected from the patient referred for *in vitro* fertilization (IVF) led to reduced fertility of oocytes.[22] Since fluorescence increase relative to CMA3 is an indicator of protamine deficiency in sperm nucleus chromatin, this study aimed to investigate the ultrastructure difference of sperm nucleus in samples with high and low CMA3 samples.

MATERIALS AND METHODS

In the samples, which had very high or very low fluorescence after CMA3 staining, the patients were called and their semen samples were collected in order to ensure high or low percentage of CMA3. The process of sample preparing for transmission electron microscope (Zeiss Company, Germany) was done in the following way. The samples of five high fluorescence and five low fluorescence cases were prepared. For pre- and postfixation, sperm samples were kept in 5% glutaraldehyde (Taab, England) for 2 h in refrigerator and in 1% osmium tetroxide for 1 h in refrigerator and 2 h at room temperature, respectively. After centrifugation, they were added to cooling 2% agar — agar solution for infiltration and becoming molded. Then, the samples were cleared for 30 min

in ethanol — acetone solution and 30 min in pure acetone. Then, resin embedding process was performed so that acetone in the previous test tube was discarded and 3:1 ratio of acetone and resin mixture was added instead and left for one night. Then, the samples were transmitted and kept in 1:1 acetone — resin mixture for 8-10 h, 1:3 acetone — resin mixture for one night, and finally pure resin for 8-12 h, respectively. Next, the samples were molded in resin and incubated at 60°C for 48-72 h; thus, resin was polymerized and hardened. Using a cutting device, ultrathin cuts of about 100 nm were prepared from the molded samples and they were collected on mesh grids. Uranyl acetate and lead citrate were used to stain the samples. Then, the cuts were examined with transmission electron microscope. Except for the mentioned cases, all the used materials were purchased from Sigma Company (Sigma, St. Loius, MO, USA).

RESULTS

In investigating different sections of the samples belonging to low fluorescence group, sperms showed a normal view[2] [Figure 1]. Nucleus occupies a main part of head and sperm head is oval in transverse sections and pyramidal in longitudinal sections. It means that head is narrowed from its base to the apex. Also, the anterior two-thirds of nucleus is covered by acrosome. Acrosome is located between plasma membrane of head and nucleus. Nucleus consists of a smooth and condensed carotenoid mass and occasionally vacuoles are observed in them (condensed structure of nucleus results from severe physical and chemical changes in the late stages of spermiogenesis. These changes include RNA removal, replacing somatic histones with protamine and gradual changes of granular chromatin in spermatid nucleus). Intact membrane of sperm together with two-layered acrosome can be seen in this figure. In addition, membrane of sperm nucleus is also intact. The zone below acrosome is also uniform.

In the samples related to high fluorescence group [Figure 2], numerous abnormal sperms were discovered. Nucleus chromatin of these sperms often indicates different degrees of insufficient condensation in chromatin. In sperms with insufficient condensation of chromatin, it is observed as scattered masses and thus such lesions are often called immature chromatin. Sometimes, these lesions are accompanied by defective acrosome as lack of uniformity or thinning.

In Figures 2 and 3, sperm chromatin is as scattered masses. There are large spaces between these masses and several small and large vacuoles are also observed in them. In addition to the above cases,

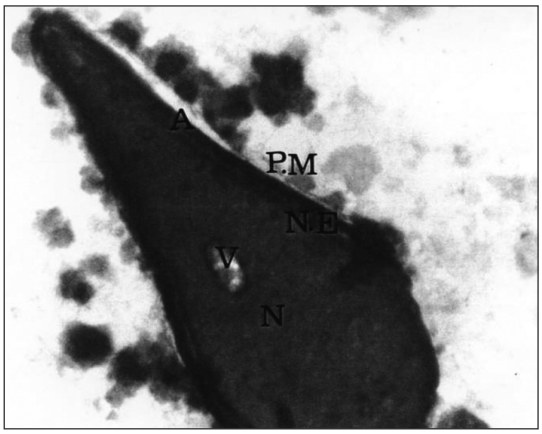

Figure 1: SEM image of a cross section of a normal sperm head belonging to the group with low fluorescence rate. Nucleus is observed with uniform and condensed chromatin in this image (magnification of ×30000)

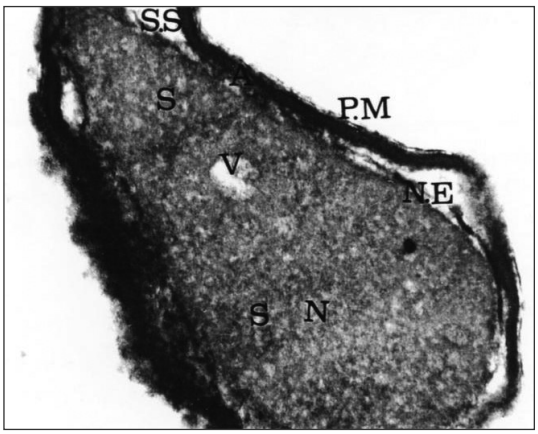

Figure 2: SEM image of a cross section of a normal sperm head belonging to the group with high fluorescence rate. Nucleus is observed with insufficiently condensed chromatin and several spaces (S). The sperm plasma membrane (PM) and nuclear envelope (NE) are irregular. In addition, acrosome (A) and subacrosome space (SS) do not have uniform thicknesses in all places (magnification of ×20000)

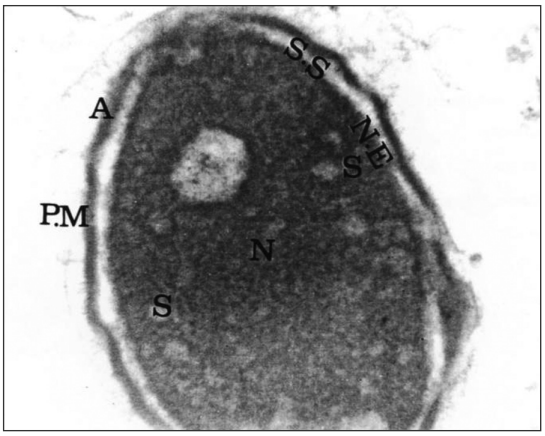

Figure 3: SEM image of a longitudinal section of an abnormal sperm head belonging to the group with high fluorescence rate. Nucleus is observed with insufficiently condensed chromatin and irregular sperm plasma membrane (PM), acrosome (A) and nuclear envelope (NE). Acrosome (A) and subacrosome space (SS) do not have uniform thicknesses in all places (magnification of ×20000)

sperm membrane and nucleus membrane lose their continuity at some points of some cross sections. Acrosome does not show uniform thickness and is not regular in all places.

Based on the observations, most sperms belonging to the group with low fluorescence rate were normal, and abnormal sperms were scarcely observed. In contrast, in the group with high fluorescence rate, most sperms were abnormal and changes were mainly related to nucleus and in the form of insufficient chromatin condensation. Besides insufficient condensation of chromatin, nuclear vacuoles, and lacunae also existed in some sperms. Of course, along with these nuclear changes, acrosome and membrane lesions were sometimes observed. These lesions can indicate the direct relationship between staining rate and occurrences of structural and ultrastructural disorders, in particular in sperm nucleus and other components of its head.

DISCUSSION

Natural trend of spermiogenesis is essential for producing healthy sperms. One of the pivotal events in spermiogenesis is replacement of chromatin proteins or histones with protamines, which provides the possibility of a new structure in sperm nucleus. This new structure leads to extreme condensation in nucleus and protects sperm DNA against invasion of nuclease and protease.[23] In addition, such a structure facilitates sperm transfer and fertilization. Due to the condensation produced by protamines, changes in their amount or their absence can cause abnormalities in condensation of sperm nucleus and consequently affect sperm quality and its ability in fertilizing an ovum.[24] Such an abnormality indicates some problems at molecular level such as disorder in protamine 1:protamine 2 ratio,[25-27] DNA strand breaks and DNA denaturation,[28-31] single-strand DNA,[15,32] and high amounts of histone.[33-35] Besides, such abnormalities may make the sperms more susceptible to external factors, which may exacerbate the sperm damage.[36]

The binding manner of protamines to DNA in sperm has been shown by different models including Balhorn model.[37] According to this model, poly-arginine area of protamine binds to the minor groove of DNA and neutralizes phosphodiester bonds of DNA. When the sperm enters the ovum, its chromatin is released and transformed to male pronucleus, which is capable of DNA transcription. This process requires a decrease in disulfide bonds (S — S) in protamine and replacement of histone with protamines during the non-condensation period.[38] As mentioned before, protamines bind along the length of minor groove of DNA.[39,40] Moreover, fluorochrome CMA3 also binds to the minor groove of DNA and this issue creates a competition with protamines. The results from the present and other studies suggest that protamine deficiency in sperm nucleus and consequently high yellow or red fluorescence rate in semen samples could decrease IVF and intracytoplasmic sperm injection.[22,41-44] Tavalaee *et al*. showed that fertility rate and amount of hollow spaces (amount of acrosome reaction) had a significant relationship with CMA3 fluorescence rate.[45] The study by Gill-Sharma *et al*. indicated that testosterone and follicle stimulating hormone deficiency caused deficiency in the amount of protamine 1 and decreased chromatin condensation of sperm nucleus taken from rats' epididymal head.

According to electron microscopic studies, these deficiencies could also cause ultrastructural changes in nucleus membrane.[46] In another study, these researchers also suggested that such rats with low protamine sperms, which suffer from disorder of chromatin density consequently, had low fertility.[47] Nasr-Esfahani *et al*. suggested that, by injecting sperm with insufficient condensation into the ovum during the non-condensing process of sperm nuclei, sperms with prematurely condensed chromatin (PCC) were produced in the ovum.[48]

This study is the first to work on examining and comparing ultrastructure of sperm in patients with high and low fluorescence. According to electron microscopic studies, most sperms of the group with low fluorescence rate had normal view and abnormal sperms were rarely observed. In the group with high fluorescence rate, most sperms were abnormal and had insufficient chromatin condensation; also, numerous cases of nuclear lacunae were observed. In some cases, acrosome and membrane lesions were observed. Previously, while investigating semen of teratozoospermia patients, Perdix *et al*. discovered some vacuoles, which were occupying more than 13% of head zone. In such sperms, chromatin condensation is usually low and abnormal acrosomes are also observed in some of them.[49] Thus, high fluorescence rate with CMA3 or in fact protamine deficiency in sperm nucleus leads to insufficient condensation of chromatin in sperm nucleus in microscopic view and existence of nuclear holes or lacunae and acrosome lesions and changes in cell and nucleus membrane.

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