Original article

Evaluation of *in vitro* sperm nuclear chromatin decondensation among different subgroups of infertile males in Mysore, India

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Background: Infertility is a condition associated with multiple etiologies. Sperm nuclear chromatin decondensation is one of the important events that occur during fertilization. Abnormal spermatogenesis leads to improper protamine package and chromatin condensation. The aim of the study was to analyze and understand the levels of fertilization capacity and nuclear stability of the spermatozoa in different infertile subgroups. **Material and Methods:** A total of 65 infertile males and 24 fertile males were employed in the study. Infertile subjects were classified into different groups according to the World Health Organization (WHO) protocol. In this study, *in vitro* nuclear chromatin decondensation status was assessed in different subgroups of infertile males. The obtained data was then statistically analyzed. **Results:** Decreased sperm chromatin decondensation was observed in different infertile subgroups compared to the control group ($p < 0.05$). Spermatozoa with swollen head indicated a positive response and unswollen head indicated a negative response. **Conclusion:** This study asserts that abnormal nuclear decondensation is a potential factor that diminishes the fertilizing capacity of the sperms among different subgroups of infertile males.

Key words: Nuclear Chromatin Decondensation, Protamines, DNA Damage, Infertility.

INTRODUCTION

Male infertility is generally characterized by dysfunctions in processes of adhesion, penetration, and fusion of sperm with oocyte.[1] During spermatogenesis, the haploid round spermatids undergo an elongation phase, cytoplasmic droplet rejection, and eventually differentiate into mature spermatozoa.[2] The formation of mature spermatozoa is a unique process involving a series of meiotic and mitotic changes in the cytoplasmic architecture, replacement of somatic cell-like histones with transition proteins, and the addition of protamines leading to a highly packaged chromatin.[3] The sperm chromatin is normally a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins. This condensed and insoluble nature protects the genetic integrity and facilitates the transport of the paternal genome through female reproductive tract.[4] Several studies have demonstrated that failure of fertilization can be attributed to the defects which lie in chromatin decondensation in infertile cases.[5-7] During spermiogenesis, the structure of chromatin is permanently modified. The first step of this process takes place in haploid round spermatids and includes replacement of somatic histones by transition proteins 1 and 2 (TNP1 and TNP2). In the phase of elongated spermatids, TNP1 and TNP2 are replaced by protamines. After binding of protamines to nuclear sperm chromatin, the process of gene transcription is completely inactivated.[8] An expression of protamines is essential for proper fertilization. In this study, an attempt was made to compare the *in vitro* sperm nuclear chromatin decondensation between fertile and infertile males.

MATERIALS AND METHODS

A total of 65 infertile males who referred to the Mediwave IVF and Fertility Hospital (Mysore, India) for further evaluation and treatment were recruited as subjects for this study. On the other hand, 24 males with proven fertility (normozoospermic) was included as controls. Institutional ethical clearance was taken from university ethical clearance committee and written informed consent letters were obtained from all participants before entering the study.

Semen collection and semen analysis:

The semen samples were collected from the infertile subjects as well as the control group through masturbation after 3-5 days of ejaculatory abstinence. The samples were collected in a sterile plastic container in a room especially provided for this pur-

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pose by following the World Health Organization (WHO) guidelines.[9] The collected semen samples were allowed to liquefy at 37°C for 30 minutes and were analyzed within 1 hour after collection. Physical examination such as liquefaction time, color, odor, and pH were recorded after 30 minutes. Basic microscopic examination was carried out to record the count, vitality, density, morphology, and motility of the sperm according to the WHO guidelines.[9]

Nuclear chromatin decondensation (NCD) test:

NCD test was performed by the method described by Gopalkrishnan.[10] Semen samples were diluted with ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) mixture and incubated with equal volume of glutaraldehyde/borate buffer at 37ºC for 60 minutes. Then, 10 microliters of the incubated mixture were placed on a clean glass slide and 200 spermatozoa were examined under a microscope using a 40x objective. Spermatozoa showing swollen heads indicated proper NCD ability which facilitated normal fertilization.

Statistical analysis:

The obtained values were recorded and subjected to statistical analyses using the statistical software called Prism3.0. The results were expressed as mean and standard error. The mean values of the groups were analyzed by analysis of variance (ANOVA). Correlation coefficients were significant at $p < 0.05$.

RESULTS

The present study demonstrated 5 different infertile conditions. Table 1 shows the spermiogram which includes sperm count, viability, motility, and semen fluid volume. Oligospermia and oligoasthenoteratozoospermic (OAT) cases showed decreased sperm count. The asthenospermic condition revealed decreased semen volume (1.2 ± 0.4) compared to the controls. In fact, OAT and oligospermic conditions were accompanied with less sperm viability which was followed by asthenospermia and teratozoospermia. Decreased motility was recorded in asthenospermic (25.4 ± 7) and oligoasthenoteratozoospermic (17 ± 3) conditions. Figure 1 depicts the response of sperms to NCD test in different infertile conditions. Decreased level of NCD was observed in OAT condition, oligospermia, and idiopathic conditions whereas asthenospermic cases presented nearer values to the control groups. Statistical analysis (ANOVA) recorded evident significant variation ($p < 0.05$) between different infertility conditions and the control group for NCD test. The response of spermatozoa for NCD test in different infertile conditions and the control group in Figure 2.

Table 1. Spermiogram details of different infertile conditions

Condition	n (%)	Count (15 millions/ml)	Volume (1.5 - 4.5ml)	Viability (50%)	Motility (50%)
Oligospermia	23 (35.9%)	11.7 ± 1.5	2.0 ± 0.2	56.3 ± 3.7	55.8 ± 4.0
Teratozoospermia	19 (29.6%)	47.2 ± 4.7	2.5 ± 0.3	68.3 ± 4.3	60.9 ± 2.2
Asthenospermia	5 (7.8%)	43.8 ± 4.4	1.2 ± 0.4	65.8 ± 6.7	25.4 ± 7.0
Idiopathic condition	9 (14.0%)	71.8 ± 18	2.0 ± 0.2	72.6 ± 4.1	65.3 ± 3.3
Oligoasthenoteratozoospermia	9 (14.0%)	7.8 ± 1.9	2.0 ± 0.3	41 ± 6.3	17.0 ± 3
Control Group	24	89 ± 3.5	2.3 ± 0.1	72.8 ± 3.3	60.0 ± 3.4

Values are expressed as mean ± standard error (SE).

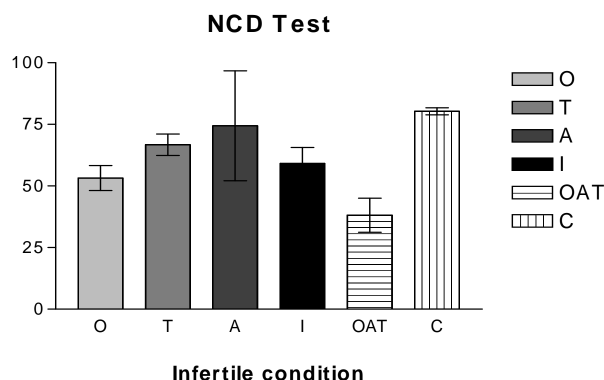

Figure 1. Response of spermatozoa in different infertile conditions for nuclear chromatin decondensation test (one-way analysis of variance: $p < 0.05$; F = 10.42; r2 = 0.38)

O: Oligosepermia, T: Teratozoospermia, A: Asthenospermia, I: Idiopathic, OAT: Oligoasthenoteratozoospermia, C: Controle

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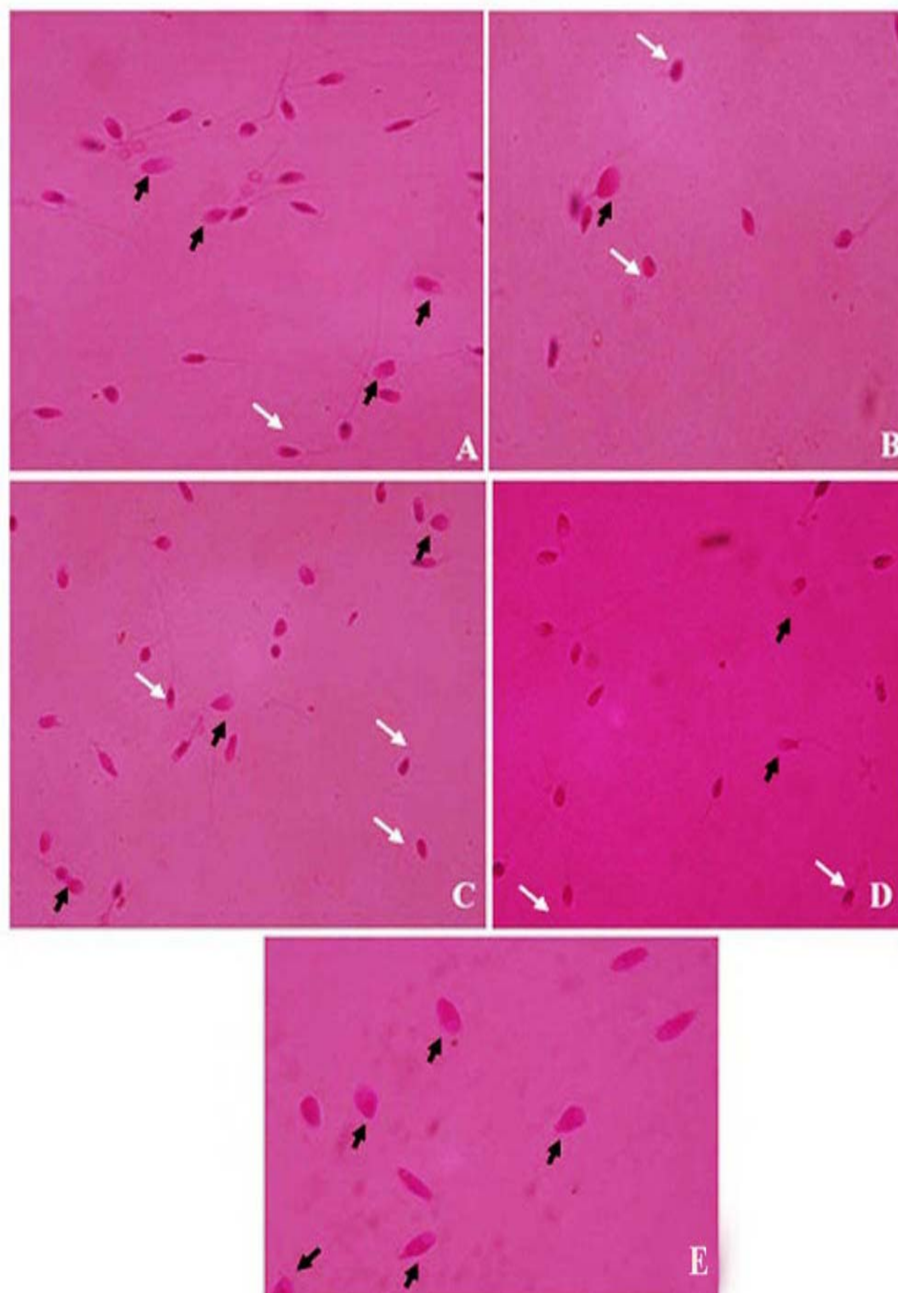

Figure 2. Sperm nuclear chromatin decondensation (NCD) response in different infertile subgroups (White arrows indicate negative response and black arrows indicate positive response.)
Plate A: NCD response in teratozoospermic cases
Plate B: NCD response in oligospermic cases
Plate C: NCD response in asthenospermic cases
Plate D: NCD response in oligoasthenoteratozoospermic cases
Plate E: NCD response in the control group

DISCUSSION

Successful fertilization and formation of pronucleus depends on the sperm NCD ability in the oocyte. The failure of sperm decondensation in the oocyte due to sperm abnormalities is unrecognizable by conventional semen analysis.[11] This study demonstrated reduced sperm chromatin decondensation in different infertile subgroups compared to the control group. Cho et al. established that a subset of infertile men (5-15%) pos-

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sessed complete protamine deficiency. By genetic analysis, they confirmed that it was due to a mutation of protamine gene cluster.[12] Environmental stress, gene mutations, and chromosomal abnormalities can disturb the highly refined biochemical events that occur during spermatogenesis, and this can ultimately lead to an abnormal chromatin structure.[13] In our study, oligospermia and OAT condition accompanied with very less chromatin decondensation which indicates abnormal protamine package and susceptibility to DNA damage. Since such protamines are small, i.e. only half of the size of the core histones which are extremely basic, around 55-70% of the amino acids among them are arginine. Furthermore, the sperm protamines also contain numerous cysteine residues, which are used to generate disulfide cross-links between adjacent protamine molecules during chromatin condensation.[14] The 4 levels of organization for packaging in the spermatozoa are (i) chromosomal anchoring, which refers to the attachment of the DNA to the nuclear annulus; (ii) formation of DNA loop domains as the DNA attaches to the newly added nuclear matrix; (iii) replacement of histones by protamines, which condenses the DNA into compact; and (iv) chromosomal positioning.[15] DNA stability of sperm also depends on the amount and ratio of disulfide bonds (S-S), non-covalent bonds between zinc and sulfhydryl groups (SH=Zn=SH), and free thiol group which reflects the epididymal maturation and more or less normal function of male genital glands. Moreover, the ability of NCD depends on the SH=Zn=SH bonds.

In this study, asthenospermic and teratozoospermic conditions showed positive response nearer to normal. Immotility in asthenospermic condition could hence be because of the mitochondrial abnormality. Although we expected negative response of teratozoospermic condition to NCD due to abnormal shaped sperm heads, our results were positive which indicates that DNA damages were not evident in the teratozoospermic subjects assessed in this study. Other infertile conditions in our study subjects showed poor or no response in NCD test which could be due to low levels of protamines. Low levels of protamine (PN1, PN2) may lead to accumulation of DNA damages in sperm, morphological abnormalities, initiation of apoptotic pathway, inactivation of mitochondria, and decreased sperm motility.[16] Several studies have suggested that poor chromatin packaging and/or damaged DNA may contribute to failure of sperm decondensation after intracytoplasmic sperm injection (ICSI) and thus failure of fertilization.[7] Hence, NCD of spermatozoa and subsequent male pronucleus formation are essential for fertilization and normal embryonic development.

CONCLUSIONS

The knowledge of sperm NCD is important in the field of assisted reproductive technique (ART). This study helped in examining the levels of fertilization capacity and nuclear stability of the spermatozoa in different subgroups of infertile population.

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