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Andrology

Cytogenetic Analysis of Sperm Nucleous Components of Iranian Normal and Sub-Fertile Individuals Using Zona Free Hamster Oocytes

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***Purpose*:** The purpose of this study was to investigate whether infertility is affected by sperm chromatin and cytogenetic abnormalities. To this purpose, the frequency of sperm premature chromosome condensation (PCC) induction and numerical chromosome abnormalities in the sperm of normal and sub-fertile men were analyzed. PCC rate was studied for evaluating the role of sperm chromatin abnormalities in the process of nuclear decondensation.
***Design*:** Controlled prospective study.
***Setting*:** Infertility Genetics Department, Royan Institute.
***Patient*:** Sub-fertile males who were referred for infertility treatment and sperm cytogenetical studies.
***Methods*:** Hamster oocytes were retrieved after super ovulation by PMSG and HCG injection. Following treatment with Hyaloronidase, zona was removed by trypsin digestion. Sperms were classified according to the morphology, movement and counts and then processed by swim up method. After capacitation, zona-free oocytes were incubated with sperms, and then transferred to fresh media containing colcemid. Slides were prepared using Tarkowskie's standard air-drying technique. Oocytes were analyzed using $\times$ 1000 microscope after staining in 5% of Giemsa.
***Main outcome measure*:** The incidence of sperm aneuploidy, PCC and penetration rate in three groups were determined.
***Results*:** Regarding the PCC rate, a significantly higher frequency was found in infertile patients. ($P < 0.001$). The frequency of PCC in oligosperm samples was 36% compared to 19.37% in normal group. A higher frequency of numerical chromosome abnormalities was found in infertile patients. The rate of these abnormalities was 5.6% in normal group and 18.5% in oligospermic samples. Despite the considerable difference between those frequencies, this difference is not significant. ($P > 0.05$)
***Conclusions*:** From the results it can be concluded that, formation of sperm PCC is a major cause of failed fertilization in individuals with sperm abnormalities. PCC may form due to chromatin abnormalities, improper DNA packing, chromosomal abnormalities and penetration delay of sperm. Also this may be involved in the etiology of some cases of idiopathic infertility. About numerical chromosome abnormalities although the differences are not significant, there is an association between sperm numerical chromosome abnormalities and male infertility. These abnormalities can be originated from meiotic process in spermatogenesis.

KEY WORDS: Chromosome abnormalities; hamster zona-free oocyte; human sperm; in-vitro fertilization.

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INTRODUCTION

It has been possible to analyze human sperm chromosome complements for the last decade since the discovery that human sperm would penetrate hamster eggs and form pronuclear chromosomes. Studies on normal and infertile men have provided information on the frequency and type of chromosomal and cytogenetical abnormalities in human sperms (1). Humans have an exceedingly high frequency of chromosomal and cytogenesis abnormalities which are manifested as pregnancy loss and the birth of children with physical and mental handicaps (2).

Infertility, the inability to produce live offsprings after attempting to do so for one year, affects 15% of couples, with approximately one-half of infertility cases due to male factors. The majority of male factor infertility is due to oligospermia, asthenospermia or oligoasthenospermia. Recently, assisted reproductive techniques such as intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) are the ways that allow infertile men to have children (3). It has been established that there is an increase in the frequency of constitutional chromosomal abnormalities in the infertile male population compared to that seen in the normal men (4). To understand the mechanisms that affect the production of chromosome abnormalities, it is necessary to study human gametes directly. The great majority of errors occur during meiosis and analysis at different stages of meiosis will lead to the mechanisms of these problems (5). After aneuploidy, sperm PCC is the next most important cause of fertilization failure (6).

In general, fusion of a mitotic and interphase cell will induce PCC in the interphase nucleus. When a cell whose chromosomes are in metaphase, like a mature oocyte, is fused with an interphase cell, the nuclear membrane of the interphase nucleus dissolved, its chromatin condense into chromosomes, a phenomenon observed by Johnson and Rao (1970) (7) in experimentally fused cells at different stages of the cell cycle. Different degrees of PCC can be distinguished, but only few investigators have provided a detailed classification (6, 8–10).

The latter can be in different stages (G1-S or G2) of the cell cycle and this determines the morphology of prematurely condensed chromosomes: G1-PCC is characterized by single chromatids, S-PCC appears pulverized and G2-PCC is represented by chromosomes consisting of two chromatids (9).

A similar observation has been made in oocytes remaining unfertilized after IVF (8) and ICSI (11).

It has been well established in normal IVF that cytoplasmic chromosome condensing factors of an oocyte arrested at metaphase II can induce a premature chromosome condensation (PCC) in the penetrating sperm nucleus (8). Yet many oocytes contain partially decondensed sperm chromatin, indicating a correct performance of penetration. Following the entrance of sperm into an oocyte, the oocyte becomes activated which results in completion of meiosis and formation of both male and female pronuclei. However, under some circumstances, although the sperm is within the oocyte, fertilization fails to occur. The oocyte remains at metaphase II stage while the sperm chromatin undergoes premature chromosomal condensation, separate from the oocyte chromosomes. It is assumed that these oocytes do not become activated and remains arrested at MII, therefore still posses chromosome condensing factors inducing a PCC of the sperm nucleus (10). PCC can be the result of many problems in oocyte or sperm, such as decreasing the Maturating Promoting Factors (MPF) in oocytes (12,13), chromatin anomalies (14) and improper DNA packaging and chromosomal abnormalities in sperm (15). Some studies have shown that protamine deficiency independently effect fertilization outcome in IVF and ICSI cases (14,16).

The objective of this cytogenetic-cytological study was to elucidate whether infertility is affected by PCC induction and numerical chromosome abnormalities rate in the sperm of normal and sub-fertile men.

MATERIALS AND METHODS

Hamster Care and Super Ovulation

Female golden hamsters purchased from Pastor Institute (Tehran-Iran), were transferred to animal house of the laboratory at 5–7 weeks of age. They kept at 23°C and 14 h of light and 10 h of darkness. The hamsters were given a few days to adjust to these conditions. They were superovulated with an intraperitoneal injection of 25–30 IU of PMSG (Intervet), followed by injection of 25–30 IU of HCG (Organon) after 48–56 h of first injection. The cumulus cell mass containing eggs is in the oviduct 15–17 h later. Each hamster yield approximately 25–50 eggs.

Media

Hams-F10 medium (Biochrom.K.G) with some modifications is used for sperm preparation, capacitation, egg preparation and IVF.

This media was supplemented with 15% fetal bovine serum (Sigma). Media was incubated in 5% CO_2 and 37°C. (The buffer in the media will lower the pH slightly at 37°C).

Preparation of Sperm

The semen samples were collected in sterile containers processed after liquification (10–20 min at 37°C). Total sperm counts, number of sperms/ml, motility, forward progression were determined. Sperm samples divided in 3 groups according to WHO criteria (Normal, Oligosperms, Asthenosperms). For each group 30 samples were analyzed.

Sperms were processed by *Swim-Up* technique and were incubated for 5 h in Hams-F10 medium in 37°C and CO_2 incubator for capacitation.

The capacitation time for normal and oligosperm was 3 h but asthenosperm samples needed more than the other groups (1–2 h more).

Preparation of Eggs

The Hamsters were sacrificed by cervical dislocation and the oviducts were punctured. The cumulus cell mass was removed and transferred to a solution of 0.1% hyaloronidase (Sigma). After washing the oocytes in fresh media, they transferred to a solution of 0.1% trypsin (Gibco) for zona removal. Then they were washed in Hams F-10 and transferred to fertilization drops containing capacitated sperms at a concentration of 10^6–10^7 per ml of medium. They were incubated for approximately 3 h (for asthenosperm samples) to allow sperm penetration to occur.

Egg Culture

After 3 h co-incubation of sperms and oocytes, the eggs were washed to remove excess sperms. Twenty to thirty eggs were scattered throughout one drop of media again and incubated overnight in 37°C and CO_2 incubator. The next morning the eggs were transferred directly to drops of F-10 containing 0.4 μg/ml colcemid (Sigma) under sterile paraffin oil for 4–7 h.

Slide Preparation

Approximately 15 eggs were transferred from colcemid solution to the hypotonic solution (1% sodium citrate) for 10 min. Three to five eggs were transferred to the center of a microscope glass slide. Then oocytes were fixed by Tarkowskie's air drying technique (17) with a slight modification. Eggs were treated with a hypotonic solution consisting 1% sodium citrate (Sigma), distilled water for 15–20 min. Sequential fixation procedure was used for eggs in three stages as follows:

1. Fix I: Consisting of methanol (Merck), glacial acetic acid (Merck) and distilled water (5:1:4) for 3 min.
2. Fix II: Consisting of methanol, glacial acetic acid (3:1) for 10 min.
3. Fix III: Consisting of methanol, glacial acetic acid and distilled water (3:1:1) for 60 s. High humidity aids in the spreading of chromosomes.

Slides were stained in 5% Giemsa solution and were analyzed using ×1000 light microscope. For chromosome study of sperms, higher magnification (×2000) was used.

Statistical Analysis

Student *t*- and χ^2 tests were used for statistical evaluation of the results.

These analysis were done with SPSS software.

RESULTS

A total of 2050 zona-free Golden Hamster oocytes were analyzed. The results are summarized in Table I and shown in Figs. 1 and 2.

From the 457 oocytes which were analyzed in normal group; 384 oocytes showed the sperm penetration (84.07%), of which 72 oocytes had sperm PCC (19.37%) (Fig. 1).

In oligosperm and asthenosperm men, for obtaining more penetration rate and study the adequate number of samples (30 people for each group); the higher number of oocytes were analyzed. For oligosperm men 788 oocytes and for asthenosperms

Table I. Results of Co-Incubation of Zona-Free Hamster Oocytes and Human Sperms

Parameters	Number of analyzed oocytes	Sperm penetration	Intact sperm head	Sperm PCC
Groups				
Normal	457	384	34	72
Oligosperms	788	568	131	204
Asthenosperms	805	423	105	296
Total	2050			

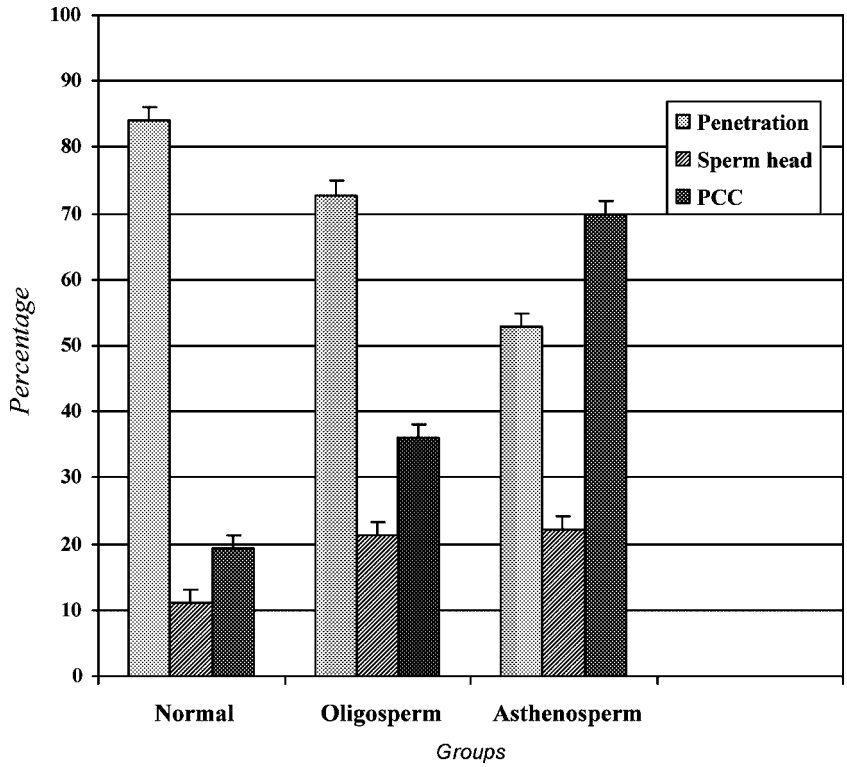

Fig. 1. Percentage of sperm PCC, intact sperm head and penetration rate of sperms in three groups.

805 oocytes were studied. In oligosperm group; sperms penetration were seen in 568 oocytes (72.86%) but 204 showed sperm PCC formation (36%). 805 oocytes were incubated with asthenosperm samples and 423 oocytes showed the evidence of sperm penetration (52.87%) but 296 oocytes had PCC of sperms (70.01%).

The statistical analysis showed that; there was a significant difference between the penetration rate of normal and oligosperm samples and asthenosperm samples ($P < 0.001$) (Fig. 1).

The frequency of PCC in normal samples was seen to be the lowest rate between these 3 groups and the rate of PCC in asthenosperm group was completely higher than normal and oligosperm samples. The differences of frequencies between groups were statistically significant from each other ($P < 0.001$) (Fig. 1).

As seen in Fig. 1, the frequency of intact sperm heads in normal individuals was 11.1%, in oligosperm samples 21.2% and in asthenosperm persons was 22.2%.

Statistical analysis showed significant difference between the rate of intact sperm heads in normal samples with oligo and astheno persons.

When studying the chromosome constituent of some samples with appropriate spreads, a higher frequency of numerical chromosome abnormalities was found in sub-fertile individuals. The rate of numerical chromosome aberrations was 5.6% in normal group and 18.5% in oligosperm samples (Fig. 2).

As our main objective of this study was to analyze the chromosomal constituents of sperms of different groups, because of high PCC rate were seen in these samples, few samples showed analyzable chromosome spreads. Therefore; despite the considerable difference in the obtained results, this difference is not significant ($P > 0.05$) which might be due to small sample size for each group.

DISCUSSION

The presence of a non-activated sperm head in the ooplasm and sperm nucleus PCC in varying degrees of condensation in samples (Table I) suggest that the successful introduction of spermatozoa into the ooplasm of a metaphase II oocyte is not a sufficient condition to achieve fertilization. However, despite the forced

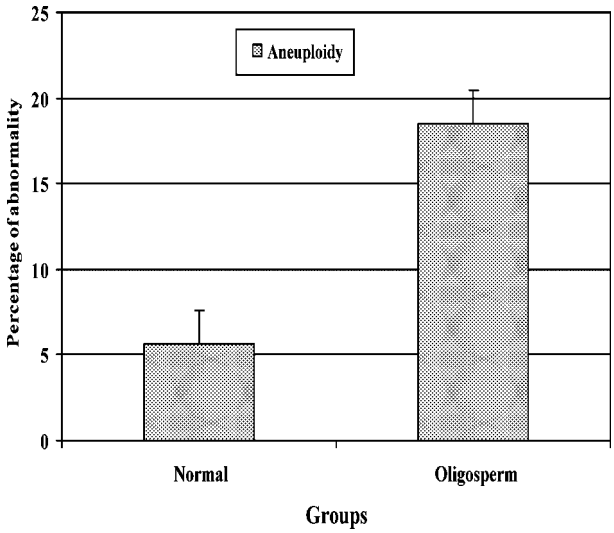

Fig. 2. Percentage of aneuploidy in normal and oligosperm groups.

intimate contact of male and female germ cell, not every penetrated oocyte will develop into a zygote (18). As seen in Table I. high frequency of intact sperm head is observed in hamster oocytes. The ooplasmic factors needed for chromatin decondensation of sperms are similar for all the sperms from normal to asthenospermic individuals. However failure to chromatin decondensation of sperms in to the ooplasmic environmental may be due to chromatin packing of sperms. Another significant observation of this study was the formation of sperm PCC at a high frequency (Fig. 1, Table I). This phenomenon was mainly reported for failed fertilized oocytes after IVF or ICSI procedures. However since PCC formation is affected by so many factors both for oocytes and sperm abnormalities. In this study we used hamster zona-free oocytes to keep the ooplasmic condition similar for all samples and to have the role of sperm abnormalities as the only variable in PCC formation.

If the oocyte does not become activated but still remains arrested at metaphase II following introduction of sperm into the ooplasm, the ooplasmic chromosome condensing factors migrate into the sperm nucleus and induce PCC (10,11,19).

Moreover, sperm chromatin abnormalities must be taken into consideration (14). Concluded that abnormal chromatin could result in decondensation failure due to a physical or mechanical inability, because patients with increased sperm DNA damage had a significantly higher number of intact spermatozoa in unfertilized oocytes as seen in the present study (Table I).

Other studies have shown that a significantly unreacted spermatozoa in unfertilized oocytes carried DNA damage compared to decondensed nuclei and that a higher rate of sperm DNA fragmentation is present in men with poor-quality semen samples. (16, 20). Thus, this is conceivable that the variation in the incidence of intact spermatozoa in different sample groups in our study reflect more sperm chromatin abnormalities in oligo and asthenosperm groups (Fig. 1).

The fertilization potential of sperm with normal chromatin organization in conventional IVF has been difficult to ascertain as results may be influenced by initiation of the acrosome reaction and sperm membrane interaction with the oocytes (21).

When using ICSI the sperm membrane-oocyte interaction is of limited importance placing more emphasis on the quality of the sperm chromatin and the ability of the oocyte to initiate decondensation and pronuclear formation. The presence of spermatozoa containing damaged DNA (20, 22, 23) and the use of ICSI to force fertilization in these patients may cause further uneasiness as to the fate of fertilized ICSI eggs.

Perreault *et al.* (1987) (24) reported the hamster sperm nuclei treated by Dithiothreitol (DTT) decondensed more rapidly when microinjected into hamster oocytes. This result in the hamster suggests that spermatozoa with a lower chromatin packaging quality should transform into pronuclei more readily and possibly more rapidly. Chromatin packaging anomalies in human spermatozoa arise because of defects in the sperm nuclei condensation mechanism such as disturbances in aberrant protamine1/protamine2 ratios (25–27) and the presence of nicks in DNA (20, 22, 23) in certain patients. These anomalies may also make these spermatozoa more susceptible to exogenous factors leading to further damages.

One of the major factors leading to chromatin packaging problems in ejaculated human spermatozoa could be due to faulty protamine deposition during spermatogenesis. Human spermatozoa from infertile patients are more likely to exhibit sperm chromatin anomalies related to the deposition of protamines (25–27).

A correlation between abnormal sperm chromatin packaging and the presence of DNA strand breaks has been shown to exist and these anomalies may arise due to faults in the mechanisms that package and protect the sperm chromatin during spermatogenesis (22, 23).

Although we do not postulate that the failure fertilization is entirely due to sperm defects, it seems

likely that poor chromatin packaging and/or damaged DNA may contribute to a failure in the decondensation process.

Finally, according to the results of our study, (Table I, Fig. 1) it seems that one of the important reasons which can describe idiopathic infertility and fertilization failure in oligosperm and asthenosperm patients is PCC induction.

In this study some sperm chromatin developed into packed metaphase chromosomes specially in normal and oligosperms samples where lower PCC rate was observed. Although; some of these chromosome spreads were analyzable, a higher frequency of aneuploidy was observed for oligospermic samples (Fig. 2), however statistical analysis showed no significant difference between the abnormality rate observed for normal and oligospermic samples. There is an association between sperm numerical chromosome abnormalities and male infertility (1, 2). These abnormalities can be originated from meiotic process in spermatogenesis. During spermatogenesis, errors in chromosome distribution (non-disjunction or anaphase lag) may occur (4, 5, 28). These errors produce aneuploid sperms. It seems that reproductive difficulties are associated intimately with cytogenetic abnormalities. The non significantly in results obtained in this study may be due to analysis of low sample size or might be due to related to the washing process of sperms (Swim-up method was used to isolate sperms which is a selective method for sperm processing).

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REFERENCES

1. Martin RH, Rademarker AW: The effect of age on the frequency of sperm chromosomal abnormalities in normal men. Am J Hum Genet 1987;41:489–492
2. Martin RH: Human sperm chromosome complements; Effect of donor age, freezing and segregation and inversion carriers. Mutation and Environment Part B; 1990;435–445
3. Testart J, Gautier C: ICSI in infertile patients with structural chromosome abnormalities. Hum Reprod 1996;11(2):2609–2612
4. Colombero LT, June JH: Incidence of sperm aneuploidy in relation to semen characteristics and assisted reproductive outcome. Fertility and Sterility 1999;72(1):90–96
5. Shi Q, Martin RH: Aneuploidy in human sperm. Cytogenet Cell Genet 2000;90:219–226
6. Mozdarani H, Aghdaei F: Cytogenetic analysis of failed fertilized oocytes from Iranian infertile women after IVF and ICSI procedures. Middle East Fertility Soc J 2001;6(3):216–225
7. Johnson RT, Rao PN; Mammalian cell fusion; Induction of premature chromosome condensation in interphase nuclei. Nature 1970;226:717–722
8. Schmiday H, Sperling K: Prematurely condensed human sperm chromosomes after (IVF). Hum Genet 1986;74:441–443
9. Schmiday H, Kentenich H: PCC after IVF. Hum Reprod 1989;4(6):989–995
10. Schmiday H, Tandler-Schneider A: Premature chromosome condensation of the sperm nucleus after ICSI. Hum Reprod 1996;11(10):2239–2245
11. Rosenbuch BE: Frequency and patterns of premature sperm chromosome condensation in oocytes facility to fertilize after ICIS. J. Assisted Reprod Genet 2000;17(5):253–259
12. Masui Y, Markert CL: Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J Exp Zool 1971;117:129–146
13. Murragy AW, Kirschner MW: Cyclin synthesis drives the early embryonic cell cycle. Nature 1989;339:275–280
14. Sakkas D, Urner, F: Sperm chromatin anomalies can influence decondensation after ICSI. Hum Reprod 1996;11(4)837–843
15. Tesarik J, Sousa M: Spermatids as gametes: indications and limitations. Hum Reprod 1998;13(3):89–107
16. Esterhuizen AD, Franken DR: Chromatin packaging as an indicator of human sperm disfunction. J Assist Reprod Genet 2000;17(9):508–514
17. Tarkowski AK: An Air-drying method for chromosome preparation from mouse eggs. Cytogenetics 1986;5:394–400
18. Palermo G, Joris H: Sperm characteristics and outcome of human assisted fertilization by sub zonal insemination and ICSI. Fertility and Sterility 1993;59:826–835
19. Dozortser D, Desutter P: Behavior of spermatozoa in human oocytes displaying no or one pronucleus after ICSI. Hum Reprod 1994;9:2134–2144
20. Bianchi PG, Mani C: Effect of DNA protamination on fluorochrome staining and in-situ nick-translation of murine and human mature spermatozoa. Biol Reprod 1993;49:1038–1043
21. Bianchi PG, Mani C: Chromatin packaging and morphology in ejaculated human spermatozoa: evidence of hidden anomalies in normal spermatozoa. Mol Hum Reprod 1996;2:139–144
22. Manicardi GC, Bianchi PG: Underprotamination and nicking of DNA in ejaculated human spermatozoa are highly related phenomena. Biol Reprod 1995;52:864–867
23. Sailer BI, Jost LK: Mammalian sperm DNA susceptibility to in-situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. J Androl 1995;16:80–87
24. Perreault SD, Naish SJ: The timing of Hamster sperm nuclear decondensation and male pronucleus formation is related to sperm nuclear disulfide bond content. Biol Reprod 1987;36:239–244
25. Balhorn R, Reed S: Aberrant protamine 1/Protamine 2 ratios in sperm of infertile human males. Experimental 1988;44:52–55
26. Belokopytova LA, Kostyleva E: Human male infertility may be due to a decrease of the protamine (P2) content of sperm chromatin. Mal Reprod Deve 1993;34:53–57
27. Yebra De, Balleson L: Complete selective absence of protamine P2 in human. J Biol Chem 1993;268:10553–10557
28. Moosani N, Cox M: Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and FISH. Fertility and Sterility 1995;64(4):811–817