

Study of Some Cytogenetic Parameters in Azoospermia and Severe-Oligospermia Patient

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Abstract

The aim of this study is to determined micronuclei index, sister chromatid exchange %, in infertile men (azospermai and severe-oligospermia) and compared with fertile men. Thirty patients (20 with azoospermia and 10 with severe oligospermia) and 15 control were studied in this project, The results show significant increase in micronuclei index, sister chromatid exchange %, in patients group compared with control.

Key words: Cytogenetic Parameters, Azoospermia, Severe Oligospermia

الخلاصة

ان الهدف من البحث لتحديد فحص دليل تكوين النويات الدقيقة والنسبة المئوية لتبادل الكروماتيدات الشقيقة لبيان الدمج في المادة الوراثية. تم دراسة 30 مريض (20 يعانون من انعدام الحيامن و10 مرضى من قلة الحيامن الشديدة) و15 رجال طبيعيين. اظهرت نتائج الدراسة وجود زيادة معنوية في النسبة المئوية للدليل الانقسام الخلوي ، تكوين النويات الدقيقة ونسبة التبادل الكروماتيدات الشقيقة للمرضى الذين يعانون من انعدام الحيامن وقلة الحيامن الشديدة مقارنة مع الرجال الطبيعيين.

Introduction

Male causes for infertility are found in about 50% of infertile couples (Pryor *et al.*, 1997; Ambasudhan *et al.*, 2003). Reduced male fertility can be a result of congenital and/or acquired abnormalities. They include infections of the genital tract, varicocele, developmental and anatomical abnormalities, endocrinopathies, immunological factors, environmental exposures, and genetic abnormalities. Frequently, however, male infertility is difficult to diagnose, and about 60-75% of cases remain idiopathic. These idiopathic cases present with no previous history associated with fertility problems and have normal findings on physical examination (Dohle *et al.*, 2004).

Congenital abnormalities include a history of testicular maldescent, karyotype abnormalities, and azoospermia (sperm concentration is $0 \times 10^6/\text{ml}$) due to congenital agenesis of the vasa deferentia (Rowe *et al.*, 2000). Karyotype abnormalities like in Klinefelter's syndrome that characterized by the presence of one or a number of extra X chromosomes, and in Down syndrome that associated with moderate to severe reduction in sperm production, also a number of rare complex genetic syndromes can affect fertility in men (Matsumoto, 1995). In case of Y-chromosome gene deletion, micro deletion are more prevalent in infertile individuals, and deletions can cause severe spermatogenic defects ranging from non obstructive azoospermia to oligozoospermia (Seshagiri, 2001).

Sister chromatid exchanges (SCE) have intrigued investigators for many years. (Taylor, 1958) used autoradiography to examine the pattern of incorporation of tritiated thymine into the DNA molecule of chromosomes. He described the apparent exchange of DNA between chromatids of metaphase chromosomes as sister chromatid exchanges. Later, investigators, (Zakharov and Egolina 1972; Latt, 1973; Korenberg and Freedlender, 1974; Perry and Wolff, 1974) developed methods without the use of autoradiography to

score SCEs in cells grown two cycles in the presence of 5-bromodeoxyuridine (BrdU) and stained with Hoechst 33258. The dye stained the unifilar chromatids (BrdU substituted in one strand of DNA while the bifilar chromatids (BrdU substituted in both strands of DNA) remained pale when viewed with fluorescence microscopy. This method was later improved by the use of Giemsa stain. It was found that Giemsa stain would combine with the Hoechst 33258 to make a permanently stained preparation not requiring the use of fluorescence microscopy. This technique became known as the fluorescence plus Giemsa (FPG) technique (Korenberg and Freedlender, 1974; Perry and Wolff, 1974).

Materials and Methods

Thirty patients (20 with azoospermia and 10 with severe oligospermia) and 15 control were studied in this project,

Blood Sampling

Five ml of blood was collected by vein puncture obtained from some Baghdad Hospitals and clinics doctors from February 2013 till May 2014. Each collected blood sample was dispensed into tubes Heparinized tubes for cytogenetic studies and obtained plasma for hormonal studies.

Cytogenetic analysis

Lymphocyte cultures were set up in the laboratory by adding 0.5 ml of heparinized blood to 4.5 ml of complete medium Quantum PBL (Proplem-based learning) supplemented with 1% L-Glutamine, 15% fetal calf serum and penicillin (100 U/ul), streptomycin (100 µg/ul) and phytohemagglutinin (PHA, 1%) (PAA, Austria) as mitogen. Cells were incubated for 72 h in a 5% CO₂ incubator. 44 h after culture initiation, cytochalasin-B (Sigma, St. Louis, MO, USA) at a final concentration of 4 µg/ml was added to the cultures. The cultures were then centrifuged at 10000 rpm for 10 min.

The pellet was resuspended in hypotonic solution (KCl, 0.087 M, PAA, Austeria) and immediately centrifuged at 10000 rpm for 10 min, and resuspended in freshly prepared, ice-cold fixative containing methanol: acetic acid (3:1) (Merck, Darmstadt, Germany), left for 20 min at room temperature. The solution was then centrifuged at 10000 rpm for 10 min, and the pellet was resuspended in freshly prepared ice-cold fixative containing methanol:acetic acid (3:1). If the solution was not clear after additional centrifugation, the last step was repeated until a clear solution was obtained. After decantation to reduce the volume to about 1 ml, the pellet was mixed with the remaining fixative and dropped from about 30 cm with a Pasteur pipette onto an ethanol washed slide; the fixative was removed by slight blowing, decantation and air-drying. Subsequently, the slides were stained in 5% Giemsa solution for 10 min. (Sahar *et al.*, 2012)

Micronuclei analysis

Micronuclei were analyzed under a blind fashion using coded slides among 1,000 binuclear cells for each sample using a light microscope with a 100× objective lens. Scoring criteria was followed as described by Fenech, (2005). All particles in the cytoplasm, with the size smaller than one-third of the main nuclei, round-shaped, and with similar staining characteristics as the main nuclei were scored as micronuclei.

Nuclear division index

The proliferation index was estimated by measuring the nuclear division index according to Lamberti *et al.* (1983).

$$NDI = [1(M1\%) + 2(M2\%) + 3(M3\%) + 4(M4\%)]/N$$

$$MN = [1(MN1) + 2(MN2) + 3(MN3) + 4(MN4)]/N$$

NDI=Nuclear division index.

M, 1, 2, 3, 4=Number of nucleate cells

MN, 1, 2, 3, 4=Number of micronucleus in cells.

N= Total number of cells.

Sister chromatid exchange

Bromodeoxyuridine:

SCE can be seen in any cell that has replicated twice in the presence of 5-bromodeoxy- uridine (BrdU) (Schvartzman and Gutierrez, 1980). It is known that SCE represent the interchange of DNA replication products, which maintain their polarity (Sasaki, 1977) at homologous loci. The precise molecular mechanism is not known.

About 10 µg per ml concentrations for each sample 24 h after initiation of cultures for two consecutive cell cycles.

Slides were stained with Hoechst stain (33258) for the analysis of cell cycle progression and sister chromatid exchange.

After 24 hr of storage in darkness, the slides were stained By a modification of the FPG (Fluorescent Plus Giemsa) technique developed by Perry and Wolff (1974). The slides were stained in 20µg/ml Hoechst 33258 for 10 min in darkness, washed in distilled water for 3 to 5 min, and then air dried and mounted in distilled water with a cover slip. The slide was then exposed to an ultraviolet lamp (wavelength of 366 nm, 115 volts, 60 Hz, 0.16 amps) for approximately 24-60 min at a distance of 4 cm, incubated in 10XSSC (standard saline citrate) at 60 C for 20 min, and then stained for 3-5 min in 3.5% Giemsa stain in phosphate buffer at pH 6.8. The stained slides were rinsed briefly in water, air dried, then viewed for SCEs by use of a 100x planar objective on a standard light microscope and translocation were analyzed.

Results and Discussion

The results obtained from micronuclei assay and sister chromatid exchange are used to detect DNA damage among the infertile men and control. A sample of 30 infertile (20 azoospermia and 10 severe oligospermia) and 15 control. The mean values of the MN (Figure 1) and SCE (Figure 2) of the two groups are given in Table 1.

Significant ($P < 0.05$) increase in the Nuclear Division Index (NDI) (Figure 3) and frequency of binucleated micronuclei , sister chromatid exchange (%) and SCE/cell values among infertile men compared with the control group.

Values in percentage indicate damage cell per 1000 binucleated for MN (Figure 4) and 30 metaphase for SCE. Values are significantly higher than the corresponding values for controls and the difference between oligospermia and azoospermia groups is not significant.

Table 1. Frequency of Micronuclei (MN) and Sister Chromatid Exchanges (SCE) in infertile men and controls

	Group	Mean±SD
Micronuclei Index	Azoospermia	2.31±0.31 A
	Severe oligospermia	2.26±0.23 A
	Control	1.24±0.15 B
Frequency of binucleated for MN %	Azoospermia	17.21±2.63 A
	Severe oligospermia	15.97±2.71 A
	Control	4.31±0.98 B
Sister chromatid Exchange %	Azoospermia	9.36±2.33 A
	Severe oligospermia	9.04±2.18 A
	Control	4.45±1.26 B
Sister chromatid Exchange/cell	Azoospermia	6.33±0.72 A
	Severe oligospermia	5.15±0.64 A
	Control	1.82±0.46 B

Different letters: Significant difference ($P \leq 0.05$) between groups.

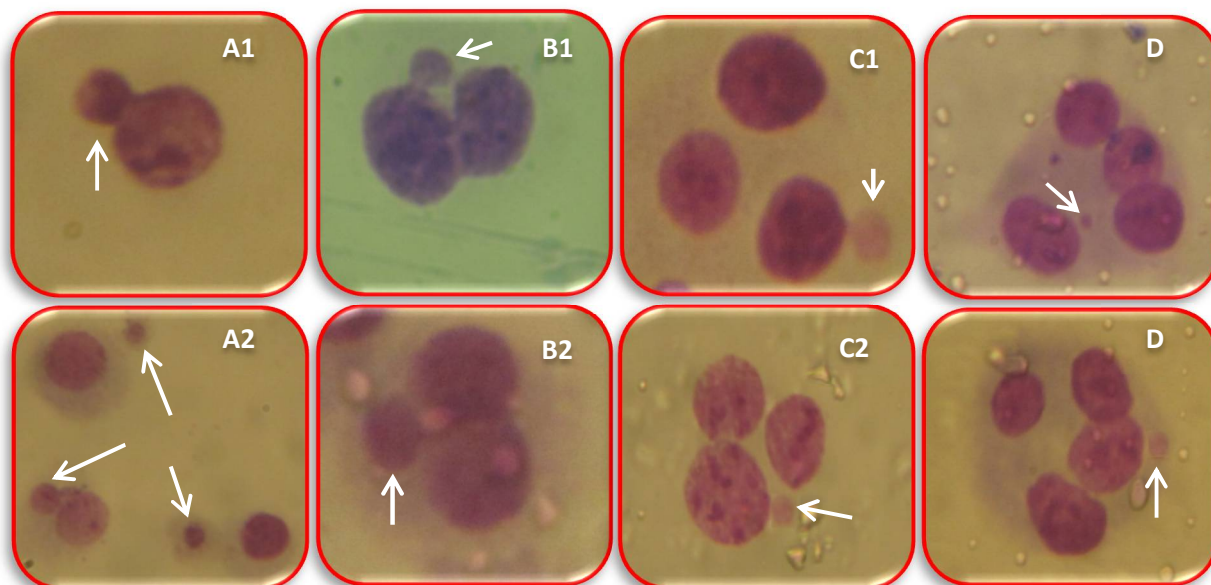


Figure 1. MNi under light microscope (100X) (A1,2) mononucleated; (B1,2) Binucleated; (C1,2) trinucleated; (D1,2) tetranucleated. Arrows are indicating on micronuclei.

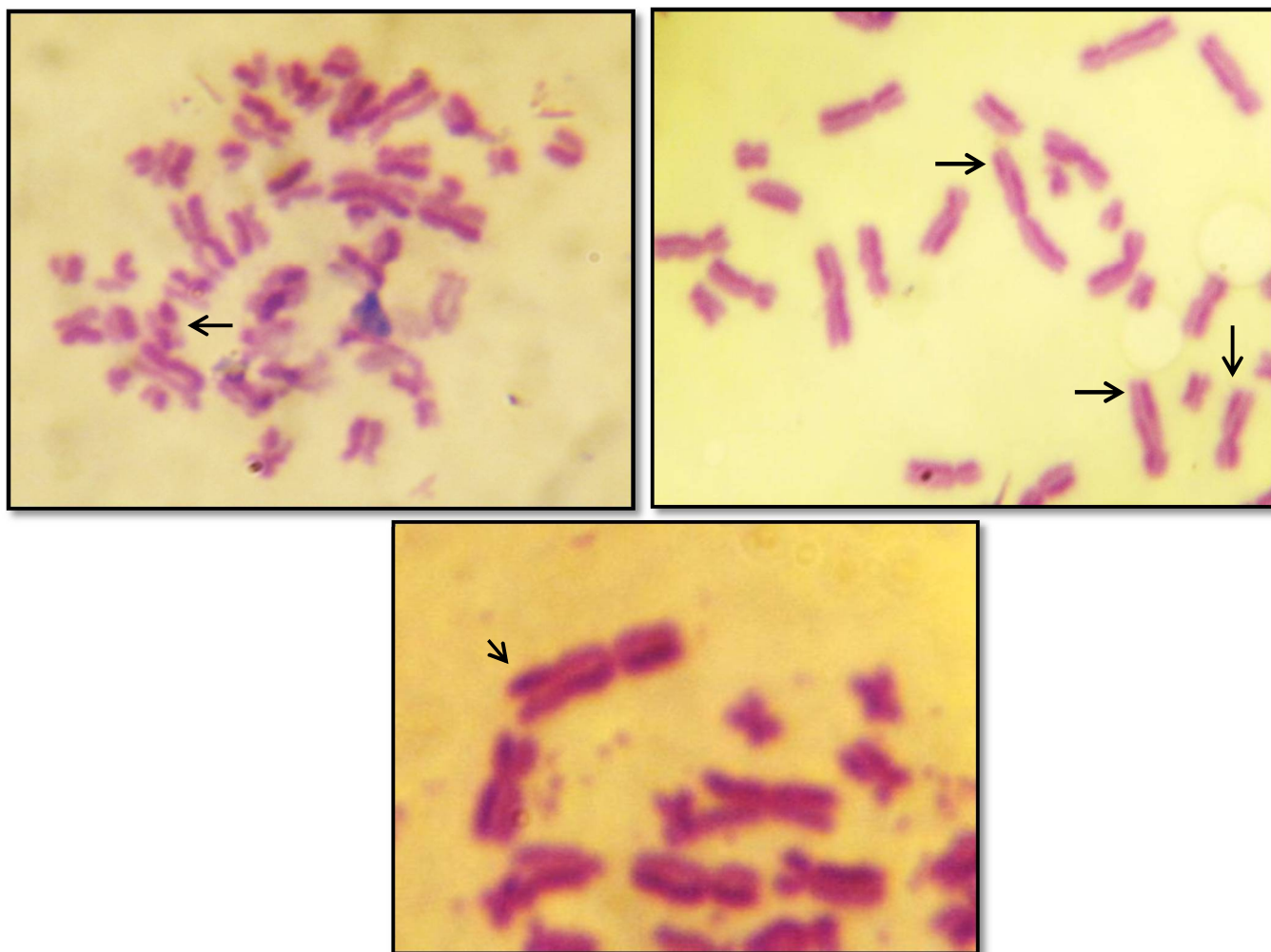


Figure 2. Sister Chromatid exchange under light microscope (100X)

Micronuclei are the result of chromosomal aberrations induced during preceding mitotic division of cells. These are from acentric fragments or lagging chromosomes induced by mutagens or clastogens such as ionizing radiation or they could be the result of non-disjunction. Chromosomal aberrations induced by clastogens are mainly arising due to DNA double strand breaks (DSB). The DSB generated by intrinsic mechanisms that could have been increased by ionizing radiation, causes adverse biological effects in cells. (Sahar *et al.*, 2012)

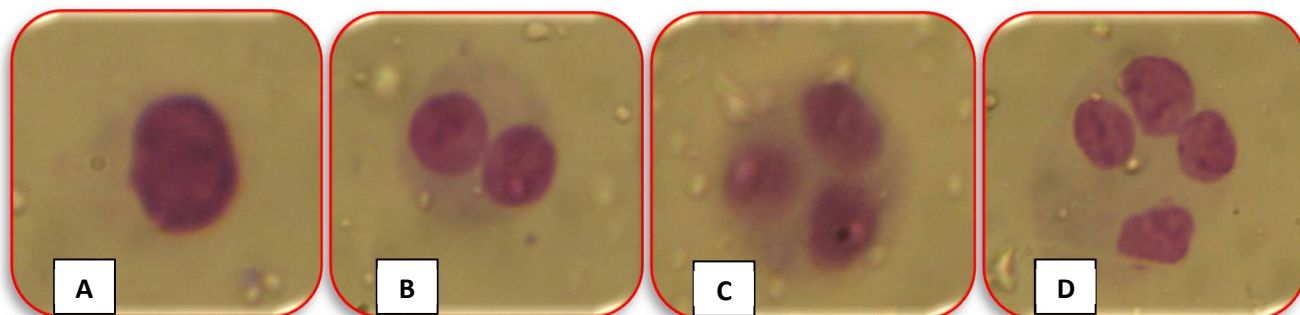


Figure 3. NDI under light microscope (100X). (A) mononucleated; (B) binucleated; (C) trinucleated; (D) tetranucleated.

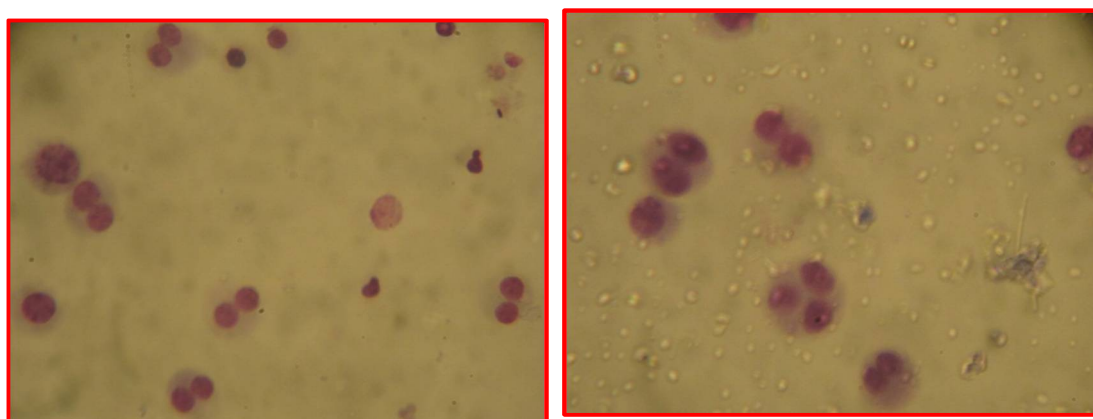


Figure 4.12. Nuclear division of lymphocyte cells from patients during counting (10X).

The chosen parameter is sister-chromatid exchanges (SCE), which is a sensitive indicator of genotoxicity (Tucker, 1993). Elevated SCE levels indicate exposure to genotoxic agents (Howell, 1991). In principle, SCE can be seen in any cell that has replicated twice in the presence of 5-bromodeoxy-uridine (BrdU) (Schvartzman and Gutierrez, 1980). It is known that SCE represent the interchange of DNA replication products, which maintain their polarity, (Sasaki, 1977) at homologous loci. The precise molecular mechanism is not known (Jayakaran and Thomas, 2005).

Sister chromatid exchange (SCE) frequency is a commonly used index of chromosomal stability in response to environmental or genetic mutagens. However, the mechanism generating cytologically detectable SCEs and, therefore, their prognostic value for chromosomal stability in mitotic cells remain unclear (Eiichiro *et al.*, 1999).

The high frequency of Y microdeletions suggests that the Y chromosome is susceptible to spontaneous loss of genetic material. Aberrant recombination events occur between areas of homologous or similar sequence repeats between X and Y chromosome or within Y chromosome itself by unbalanced sister chromatid exchanges (Yen *et al.*,

1990). The instability of the Y chromosome may be related to a high frequency of repetitive elements clustered along the length of the chromosome (Krausz and McElreavey, 1999).. Although it is very clear that microdeletions in the AZF region are responsible for spermatogenic failure, further studies are worthwhile to delineate the exact function of the genes present in AZF region and their role in spermatogenesis and fertility (Abilash *et al.*, 2010).

Symmetrical exchanges between newly replicated chromatids and their sisters can be visualized cytologically in vertebrate cells if the DNA of one chromatid is labelled with 5-bromodeoxyuridine (BUdR) during synthesis. Sister chromatid exchanges (SCEs) can be induced by various genotoxic treatments (Crossen *et al.*, 1977), suggesting that SCEs reflect a DNA repair process.

Cytological assessment of SCE levels in peripheral blood lymphocytes is used as an index of the mutagenic potential of environmental factors.

Some studies were noted that aberrant crossover events led to microdeletions in chromosomes that recombined (Kleiman *et al.*, 1999). However, for genes outside the pseudo-autosomal pairing region (PAR), recombination events between areas of homologous or similar sequence repeats on the X and Y chromosomes could also give rise to deletions or duplication events (Yen *et al.*, 1990). Furthermore, it is possible that microdeletions may be caused by aberrant or unbalanced sister chromatid exchanges (SCE). The instability of the Y chromosome may be partially related to the high frequency of repetitive elements (Graves, 1995).

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