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Micronucleus formation and Sister Chromatid Exchange in Female Lymphocytes Associated with Recurrent Spontaneous Abortion

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Abstract: This study aimed to assess possible genomic instability in women with recurrent spontaneous abortion (RSA). Sixty four blood samples from women complaining with RSA and 41 normal fertile females, who had at least one or more child, 21 of them were pregnant women while 20 non-pregnant were collected. Frequency of micronuclei (MN) in Women with RSA was statistically increased (8.66±1.74) ($p<0.05$) compared with normal fertile women either pregnant (3.83±0.74) or non-pregnant (3.61±1.02). The difference between non-pregnant and normal pregnant groups was not significant. The results obtained from SCE analysis were used to detect DNA damage among RSA women and control. The result showed a significant increase ($P<0.05$) in SCE frequency in women with RSA (8.43±2.65) compared with normal control either non-pregnant (3.85±0.97) or pregnant women (3.52±0.94), with no significant differences between them. This concluded that the frequency of MN and SCE in cultured peripheral lymphocytes can be used as a biomarker of genomic instability in somatic cells.

Key words: micronucleus, sister chromatid exchanges, recurrent abortion.

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Introduction

Recurrent spontaneous abortion (RSA) represents a significant clinical problem, which is estimated to be 1-5% of all women of reproductive age (1,2). However, the pathogenesis of RSA is complicated, and the cause in 40%–50% of the cases is not well understood (2). Genetic anatomic, endocrine, immunologic, infectious, and environmental factors have been proposed as causes of RSA (3,4). Various reports have postulated thrombophilia as a risk for RSA (5, 6).

There are many possible genetic mechanisms that can lead to infertility and a range of pregnancy complications, such as pregnancy loss, intra-uterine growth restriction, abnormal fetal development and pre-eclampsia (PE); these effects may also be due to

defective placentation. For all of these conditions, it is plausible that increases in DNA damage could diminish the viability of the embryo and the placenta that supports it. Recent studies, suggested that an increased MN frequency in lymphocytes measured using the cytokinesis-blocked micronucleus (CBMN) that assay is associated with increased risk for these conditions (7).

It is not surprising that the micronucleus assay is used for genotoxicity screening of new and promising applications in biology, medicine, and nanotechnology. For instance, genotoxic properties of carbon nanotubes which are used in electrical circuits, paper batteries, solar panels, as well as in medicine for cancer treatment can induce micronucleus formation in lymphocytes (8).

Different variants may modulate the effect of genotoxic agents on MN frequency. Mainly, they are host factors (age, gender), lifestyle (smoking, alcohol, occupation, folate, and vitamins intake), and disease susceptibility (9).

The sister chromatid exchange (SCE) are reciprocal interchanges of DNA replication products at homologous sites between 2 chromatid arms within a single chromosome in dividing cells (10).

These interchanges occur spontaneously in all cells at a low rate, and the rate of their formation increases when DNA is damaged and/or improperly repaired. In the present study an attempt was made to investigate the possible genotoxic effects among women with RSA by using MN assay and SCEs as a parameter.

Materials and Methods

Study Subjects

Patients

Sixty four Patients who had previous history of RSA, defined as three or more consecutive miscarriages, were selected in this study. The women in the study had experienced three to five miscarriages; all miscarriages had taken place during the first trimester (weeks 1-12 of pregnancy).

Controls

A positive control group was 21normal pregnant women at the first trimester, who had at least one or more child. A negative control consist of 20 fertile non pregnant with normal menstrual cycle and were not affected by pre-existing clinical conditions.

Blood Sampling

Five milliliters of blood were collected by vein puncture from all patients and control groups attending some private clinics of gynecology in Baghdad during the period between January to December 2014. Each collected blood sample was dispensed into heparinized tubes for cytogenetic 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 learing) supplemented with 1% L-Glutamine, 15% fetal calf serum and penicillin (100 U/ul), streptomycin (100µg/ul) and phytohemaglutinin

(PHA, 1%) (PAA, Austria) as mitogen. Cells were incubated for 72 h in a 5% CO2 incubator, and 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), and 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 supernatant 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 (11).

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 (12).

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 Fivision Index

The proliferation index was estimated by measuring the nuclear division index according to Lamberti (13).

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

Sister chromatid exchanges (SCEs) can be seen in any cell that has replicated twice in the presence of 5-bromodeoxy-uridine (BrdU) (14). It is known that SCE represent the interchange of DNA replication products, which maintain their polarity (15) at homologous loci.

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 (16).

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.

Statistical Analysis

Statistical analysis was performed using SPSS version 16. The frequency of MN and SCEs were compared between patients and controls using ANOVA one way.

Results and Discussion

Micronucleus Assay

Estimation of MN formation and NDI

Distributions and frequencies of micronuclei MN in lymphocytes of women with RSA and controls are summarized in (Table 1) and shown in (Figure 1).

Frequency of MN in Women with RSA was statistically increased (8.66±1.74)($P<0.05$) compared to normal fertile women either pregnant(3.83±0.74) or non-pregnant (3.61±1.02), the difference between non-pregnant and normal pregnant groups was not significant ($p>0.05$).

Table 1: Frequency of micronuclei and nuclear division index (NDI) in women with RSA and controls

Groups	Nuclear division index (Mean±SD)	Micronuclei frequency/1000cells (Mean±SD)
RSA	3.02±0.95 A	8.66±1.74 A
Non-pregnant	1.35±0.24 B	3.61±1.02 B
Normal pregnant	1.13±0.14 B	3.83±0.74 B

Different letters: Significant differences ($p<0.05$) between means of columns

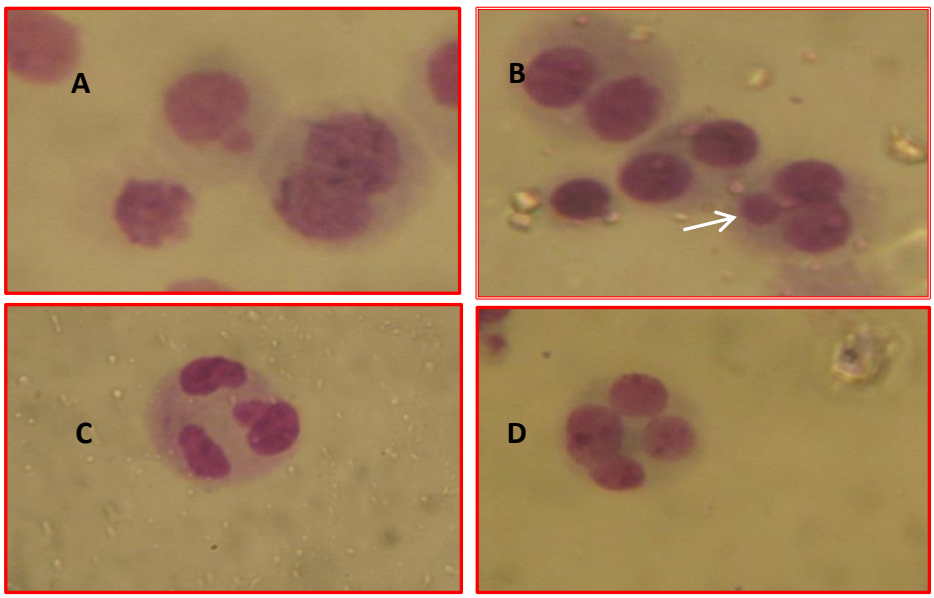

Figure 1: Micronuclei under light microscope (100X) (A) mononucleated lymphocyte; (B) binucleated lymphocyte; (C) trinucleated lymphocyte; (D) tetranucleated lymphocyte

The results obtained is correlated with other studies that suggested an increased MN frequency in lymphocytes measured using the CBMN assay and associated with increased risk for RSA. A high number of MN in lymphocytes is associated with pregnancy complications and miscarriages (7, 17). In some investigations, it has been found that couples with RSA have a higher frequency of chromosome damage in their lymphocytes by measuring MN in lymphocytes of couples with two or more spontaneous abortions (N 5 62) and compared them to fertile couples with normal pregnancy (N 5 30), they found a significantly higher frequency of MN in the infertile and spontaneous abortion couples relative to controls both when the data of each member of the couple were analysed separately as well as when the MN frequency of the couple was combined (18).

Previous studies have shown that chromosomal damage, as assessed by MN frequency, is increased in infertile couples and in those who have experienced recurrent miscarriage compared with fertile couples (19). These results suggest that MN may be a useful prognostic marker of a successful pregnancy, as they may provide an index of the genomic instability of the parental and/or fetal tissues, either because of inherited factors predisposing to genomic instability or because of nutritional inadequacies or environmental exposure to genotoxic factors causing genome damage.

Sister Chromatid Exchange

The results obtained from SCEs were used to detect DNA damage among RSA women and control. The results show significant increase ($P<0.05$) in SCE frequency in women with RSA

(8.43±2.65) compared with the normal control either non-pregnant (3.85±0.97) or pregnant women (3.52±0.94), with no significant differences (p>0.05) between them as shown in (Table 2) and (Figure 2).

Table 2: Frequency of Sister Chromatid Exchange in women with RSA and controls

Groups	Sister Chromatid Exchange % (Mean±SD)	Sister Chromatid Exchange /cells (Mean±SD)
RSA	4.53±1.09 A	8.43±2.65 A
Non-pregnant	1.63±0.45 B	3.85±0.97 B
Normal pregnant	1.36±0.38 B	3.52±0.94 B

Different letters: Significant differences (p<0.05) between means of columns

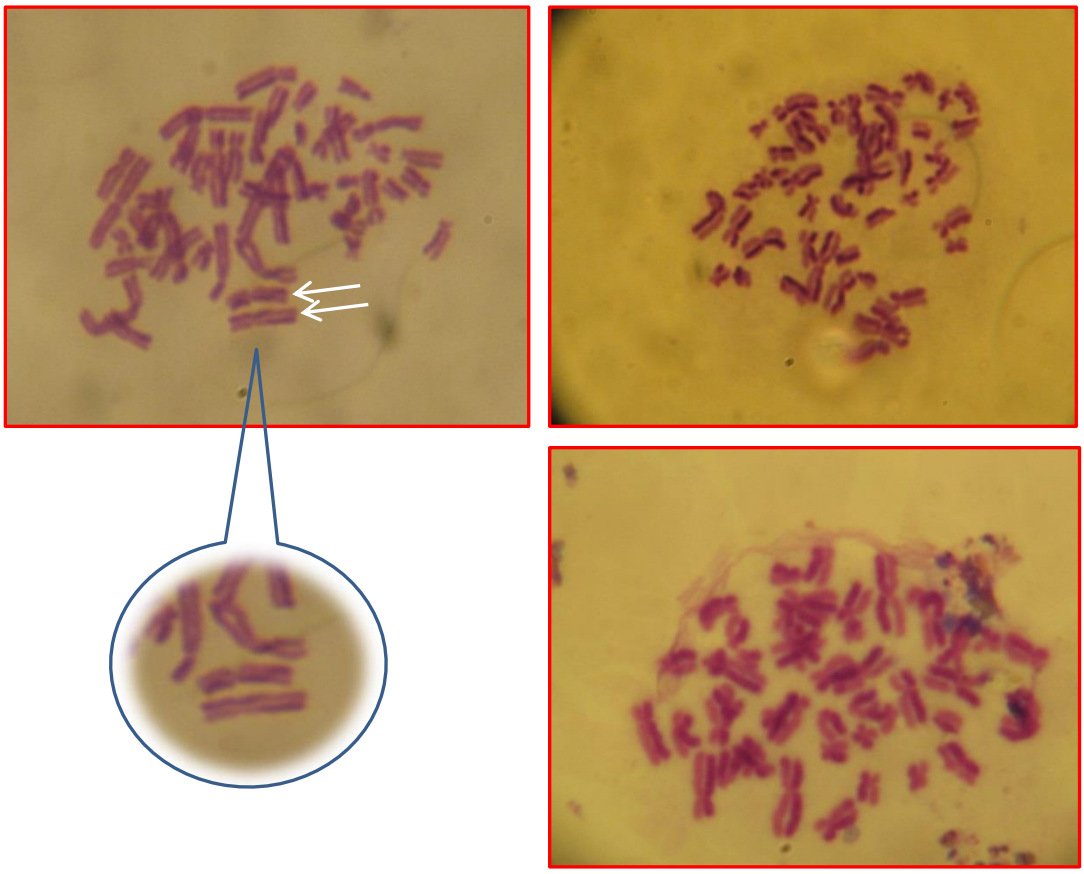

Figure 2: Photograph showing metaphase plate with sister chromatid exchange in cases with recurrent spontaneous abortion under light microscope (100X), the arrow represents chromosomes with presence of SCE

Frequency of SCE in Women with RSA was statistically increased compared to controls, while some investigations found that there is no differences in frequency of SCE in women with the history of miscarriage and matched controls (20).

On the other hands, it has been reported significantly increase in the frequencies of SCEs during pregnancy (21). The concentration of the estrogens is increased during pregnancy, which may be the possible reason of an increase in SCEs (22). Earlier studies performed on the genotoxic potential of steroids have shown to cause chromosomal damage, induction of SCEs and formation of endogenous adducts (23). Significant increase in frequencies of SCEs/cell in women use oral contraceptive may be due to the new hormonal formulations and preparations developed in an attempt to reduce the adverse effects of oral contraceptive, such as the reduction in the estrogen content (24).

The conversion of estrogen into catechol estrogens and quinines, via redox reactions causes oxidative damage to DNA (25). There are both positive as well as negative reports regarding the genotoxic effects of estrogens and synthetic progestin's (26). The SCEs are the cytological manifestation of interchanges between DNA replication products at apparently homologous loci. It is more sensitive indicator of genotoxic effects than structural aberrations (27). The results obtained in the present study are contrary to the studies performed by other workers (26, 28). The genotoxic potential of steroids is determined by the metabolic conditions in the test system and the human body. The extrapolation from the experimental data to humans is not only difficult but also complex. The most of the studies conducted for genotoxic potential of steroids involve the concentration in microgram per ml range whereas the therapeutic plasma concentration ranges from nanogram or picogram per ml (23). The present study was conducted on the women with RSA and pregnant women and the increase in SCEs may be due to the variable hormonal profile among them.

Spontaneous or base line chromosomal aberration and SCE frequency in cultured human lymphocytes may provide an index of accumulated genetic damage occurring during the life span of these cells (29). Considering that the inter individual variation could be due to different lifestyle factors, including environmental exposures or individual susceptibility factors. Previous investigations showed that in different persons treated by the same dose of mutagen, the level of chromosomal abberation was different which was explained by individual reactivity. Also the individual sensitivity to mutagen increased due to difference in metabolic activation of the mutagen and efficiency of DNA repair (25).

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