



# CYTOGENETIC AND HEMATOLOGICAL STUDY FOR SOME IRAQI WOMEN SUFFER FROM ABORTION

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## Abstract

The cytokinesis-block micronucleus assay is one of the most widely used methods to determine DNA damage caused by exposure to mutagenic, carcinogenic and environmental agents that affect women infertility. This study was aimed to assess possible genomic instability in women with recurrent spontaneous abortion (RSA). Fifty blood samples were collected from women complaining with RSA and 25 normal fertile females, who had at least one or more child. Cytogenetic study shows micronuclei frequency of peripheral blood lymphocytes is significantly decreased in comparison with that of normal pregnant women, while the nuclear division index shows no significant signs in all blood samples of aborted women.

**Key words :** Abortion, recurrent spontaneous abortion (RSA), blood samples, DNA.

## Introduction

Spontaneous Abortion (SA) or miscarriage is the one of most common complication of pregnancy, as the loss of a pregnancy before 24 weeks of gestation (Morley *et al.*, 2013). Recurrent spontaneous abortion (RSA) is a natural termination of two or more successive pregnancies before the fetus is capable of external life (20<sup>th</sup> weeks) (Pokale, 2015). Spontaneous abortion can be subdivided into threatened abortion, inevitable abortion, incomplete abortion, missed abortion, septic abortion, complete abortion, and recurrent spontaneous abortion (AL-Hilli, 2009).

There are numerous factors that might be associated with RSA, but the underlying problem often remains undetected (Larsen *et al.*, 2013; Babker *et al.*, 2015). The known causes of RSA including chromosomal and metabolic abnormalities, uterine and placental anomalies, hereditary thrombophilia, hormonal problems, nutritional and environmental factors, infection and immunologic factors (Van and Oudejans, 2013; Sharma, 2014).

In Iraq, significant number of women in recent years have shown the problem of RSA, the problem of RSA

has remarkably increased with the increase of environmental pollution in Iraq; which is believed to have an impact on this case (Fadhil and Ali, 2014). Studies have covered different aspects of abortion. Among which is the immunological cause; the results indicated that there was an association between RSA and the polymorphisms in inflammatory cytokines (IL-6, TNF- $\alpha$ ) (Jassem *et al.*, 2016). As well as, the hormonal cause; the study of which suggested that abnormal levels of one or more hormones might help in forecast RSA (AL-Barwary, 2004). Besides the microbial causes; when the fetus in uterus is infected by TORCH complex (which include *Toxoplasma gondii*, *Rubella virus*, *Cytomegalo virus* and *Herpes simplex virus*) during first half of pregnancy, this will lead to an increased opportunity of RSA (Al-Hamedi, 2012). Deformation of sperm in men is among them as well; the results show that patients male couples of females with RSA had a significant higher DNA damage comparing with healthy fertile males (AL-Ahmed, 2016). Age of the mother has been also covered; advancing maternal age has been shown to result in sub fertility and adverse pregnancy outcomes. In addition, the genetic causes; an increased incidence of fetal loss is a consequence of chromosomal abnormalities in couples

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with advanced age and these abnormalities include translocation and deletion (Jawad, 2014).

Genetic causes is common in recurrent spontaneous abortions (Pokale and Khadke, 2016). About half of all early abortion occurs due to a genetic problem within the ova or sperm (Hindi, 2012). Most of the pregnancy losses are caused by chromosomal aberrations in the fetus (Vanilla, 2015; Najafipour *et al.*, 2016). Approximately 50% to 60% of all pregnancy losses, caused by chromosomal aberrations (Tatiana *et al.*, 2016). Cytogenetic analysis is necessary in each case to confirm the diagnosis and to assess genetic implication for the family (Robberecht *et al.*, 2009). The cytogenetic analyses should be also recommended in couples with RSA because the cytogenetic results could provide important information for their genetic counseling and future genetic prevention (Petrova-Tacheva *et al.*, 2014; Najafipour *et al.*, 2016).

A micronucleus assay is one of the preferred methods for assessing chromosomal damage as a result of environmental mutagen exposure as well as a tool for genotoxicity testing (Rossnerova *et al.*, 2009). Cytokinesis - block micronucleus (CBMN) assay may provide a new approach for the early detection of women at risk of developing these late pregnancy diseases and for biomonitoring the efficacy of interventions to reduce DNA damage, which may in turn ameliorate pregnancy outcome (Fenech, 2005). Increased lymphocyte micronucleus frequency in early pregnancy is associated prospectively with pre-eclampsia and/or intrauterine growth restriction (Furness *et al.*, 2010). High number of MN in lymphocytes is associated with pregnancy complications and miscarriages (Fenech, 2011; AL-Faisal *et al.*, 2015; Mahood, 2015).

Although many factors associated with RSA were studied in Iraqi women even chromosomal changes as mentioned above, comet assay and micronuclei still have not been studied well. Therefore, this project has been proposed to do that.

## Materials and Methods

### Subjects and selection of the patients

Fifty women were used in this experiment, divided into three groups, also 25 blood samples from normal delivery women with an age range from (20 – 45) year. as the following:

- Group (I) was normal delivered pregnancy (n=25).
- Group (II) represents aborted women for the

first time during the first trimester (n=21).

- Group (III) represents repeated aborted women during the first and third trimester (n=29).

### Blood sample for cytogenetic analysis

Peripheral blood samples were collected from patient. Four ml of blood was collected by venipuncture using a disposable 5ml syringe and it is drawn into heparin tube. The same amount of blood were collected from controle. All blood samples were shipped in a cool box. The blood were culturing and assessed for micronucleus assay (MN), nuclear division index (NDI).

### Solution and Media for cytogenetic study

#### Phytohemataglutinin (PHA)

It was provided as liquid ready to use by Sigma (IAEA, 2001).

#### Antibiotic solution (Tronge, Germany)

Two types of antibiotic were prepared and used in the culture medium .One vial of penicillin (1000000 IU) was dissolved in 5 ml of distilled water, as stocks solution that concentration was (2000000IU/ml) and streptomycin (1g) was dissolved in 5 ml of distilled water, as stocks solution that concentration was (2000000µg/ml),and these were stored at -18 oC till use (IAEA, 2001).

#### Fetal calf serum

Sterile solution (Gidco, USA) (IAEA, 2001).

#### Roswell Park Memorial Medium (RPMI)-1640

RPMI-1640 medium solution (4.5 ml ready to use) which supplemented with HEPES and L- glutamine were mixed with 20% of heat inactivated fetal calf serum, 100 UI/ml of penicillin and 0.1 mg / ml of streptomycin. PH of solution was adjusted to 7.2, sterilized by filtration with Millipore filter 0.22 µm. The culture medium was divided in sterile culture tube as aliquots of 5 ml volume and kept at 40°C (Roony and Czepulkowski, 1986; Branch *et al.*, 1997).

#### Hypotonic solution (0.01 M KCl)

The hypotonic solution was prepared by dissolving 1.1175 g of potassium chloride (KCl) in 200 ml of D.W. The solution was either freshly used or stored at 4 oC and used within 4 days as a maximum duration of storage (Allen *et al.*, 1977).

#### Cytochalasin-B (Cyto-B)

This solution was prepared according to Fenech (2000). Stock solution of Cytochalasin-B was prepared by dissolved 1 mg of it with 1.6 ml of dimethyl sulfoxide (DEMSO). It was stored at -18°C till use.

### Fixative solution

The fixative was freshly made by mixing of absolute methanol and glacial acetic acid in the ratio of 3:1 (V: V) (Allen *et al.*, 1977).

### Giemsa stain solution

This solution was prepared by dissolving 1 grams of giemsa powder with 66ml of Glycerol and 66 ml of absolute methanol, stirred for two hours at 60°C on a water bath, then mixed well and filtered using Watman No.1 filter paper. The stock solution was kept in a dark bottle at room temperature till use (IAEA, 2001).

### Phosphate buffer saline (PBS)

Phosphate Buffer Saline (PBS) was prepared by dissolving the following components in 800ml distilled water, then adjusted pH to 7.2 by pH-Meter and the volume was complete to one liter with distilled water.

Sodium chloride (NaCl) 8.0 gm

Potassium chloride (KCl) 0.2 gm

Disodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>) 1.15 gm

Potassium dihydrogen phosphate (KH<sub>2</sub> PO<sub>4</sub>) 0.2 gm

The buffer was stored at 4 C. prior to any usage; PBS was warmed to 37°C (Freshney, 1994).

**Micronucleus assay and NDI** (Fenech and Morley, 1985).

#### ❖ Blood culture technique

- Under aseptic conditions, 0.5 ml of blood was added to each culture tube contain 4.5 ml of media RPMI-1640 with FCS .
- To all test tubes, 0.15 ml of PHA was added, mixed the components very well
- Tubes were sealed and incubated at 37°C for 72hours.

#### ❖ Harvesting

- After 44 hrs of incubation of lymphocyte, add 40 µL of cyto-B.
- After a 72h incubation period, samples were centrifuged for 10 min.at 1500 rpm.
- The supernatant was withdrawn by pasture pipette and the precipitated cells with a little culture medium were left in the test tube.
- The precipitate was mixed, and then 5-10 ml of warmed (37°C) Potassium chloride(KCl) (0.01 M) was gradually and gently added with mixing for 3 min.
- Samples were centrifuged for 10 min. at 1500 rpm, then the supernatant discarded.

- Few drops of the freshly made fixative were added drop wise gently mixing. Later on centrifugation performed for 10 minutes, at 1500 rpm then fixative decanted off and the process repeated for 2-3 times until the supernatant looked clear. At the final change, the cells resuspended in 1-1.5 ml of freshly made fixative.

#### ❖ Slide preparation

- To remove the fatty layer of the slide has been placed in a cleaning solution containing a mixture of 80% Methanol and 20% HCl for 24 hours, and then placed in solution of 70% Methanol for 5 hours and then washed slides with distilled water well was used immediately after drying (IAEA, 2001).
- The suspension was mixed very well by pasture pipette, 3-4 drops of cells suspension dropped onto wet clean glass slides and allowed drying at room temperature and then labeled.

#### ❖ Staining

- The slides were stained using freshly made of 2 ml giemsa stain (stock solution) and 2.5 ml Absolute methanol and 80 ml distilled water and 0.5 ml NaOH concentration 0.5%, mix content well and put the slides to be dyed and left for 20 min, and then washed slides with distilled water well ,after that left to dry at room temperature.
- Microscopic examination under oil immersion (100X) objective lens.

#### ❖ Assessment of micronucleus frequency

At least 1000 cells were scored to assess the frequency of MNi, the cells were classified as mononucleate, binucleates, trinucleates and tetranucleates. The increase in the cell number containing micronuclei is classified as a positive result. The positive result from the *in vitro* micronucleus test indicates chromosome damage or damage in the cell division apparatus. While negative results have to be confirmed (Kirsch-Volders, 1997).

#### ❖ Nuclear division index

The proliferation index was estimated by measuring the nuclear division index according to (Fenech, 2000).

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

M1, M2, M3, and M4 represent the number of cell with one, two, three and four nuclei, respectively.

N = The number of scoring cell which are at least 1000 cell.

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

MN1, MN2, MN3 and MN4 represent different number of Micronucleus from one to four respectively in bi-nucleate cell.

N = The number of bi-nucleate cell scored which are at least 1000 cell

### Results and Discussion

Results shown in table 1 illustrated a significant decrease ( $P < 0.05$ ) in group 2 ( $0.0041 \pm 0.0005$ ) compared with the control group ( $0.0063 \pm 0.0005$ ) and group 3 ( $0.0069 \pm 0.0009$ ), while there was a non-significant change between group 3 ( $0.0069 \pm 0.0009$ ) compared with the control group ( $0.0063 \pm 0.0005$ ).

In table 2, when comparing between deference groups in NDI, the table illustrated non-significant change in group 2 ( $1.591 \pm 0.02$ ) and group 3 ( $1.536 \pm 0.02$ ) compared to the control group ( $1.583 \pm 0.11$ ), so as there is non-significant change between the experimental group 2 ( $1.591 \pm 0.02$ ) and group 3 ( $1.536 \pm 0.02$ ).

Cytogenetic studies have an important role in the evaluation of couples with repeated miscarriages. The genetic factors were found to represent more than 50% of early gestation spontaneous abortion and associated with fetal chromosomal abnormalities (Purandare *et al.*, 2011). The present results and those obtained by other researchers showed that chromosomal content of couples with recurrent abortion are usually normal (Stirrat, 1990; Egozcue *et al.*, 2003).

In this study, different cytogenetic biomarkers have been used for assessment of women with repeated miscarriages. They are micronucleus assay MN, nuclear division index NDI, and comet assay. The results showed a decrease frequency of MN in peripheral lymphocytes of women with RSA.

The MN test reflects chromosomal damage (Pederser, 2009). Micronuclei (MN) are formed from lagging chromosomal fragments or whole chromosomes at anaphase which are not included in the nuclei of daughter cells. They are therefore seen as distinctly separate small spherical objects that have the same morphology and staining properties of nuclei within the cytoplasm of the daughter cells (Countryman *et al.*, 1976). Studies of mutations at MN frequency have provided insights into several aspects of somatic mutations in vivo. This includes molecular mechanisms of mutagenesis, the relationship between DNA damage and mutation, and individual susceptibility factors such as DNA repair capacity (Ward, 1988; Georgakilas, 2008). A high number of MN in lymphocytes is associated with pregnancy complications and miscarriages (Fenech, 2011; Olivera *et al.*, 2003).

Some investigations show 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. When comparing them to fertile couples with normal pregnancy, they foud 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 analyzed separately as well as when the MN frequency of the couple was combined (Trkova *et al.*, 2000). Stephano and Roberto (1999) reported that there were no significant differences in the frequency of MN found between women with a history of miscarriage and controls. Mahood (2015) that showed the frequency of micronuclei (MN) in women with RSA is statistically increased as compared to normal fertile women either pregnant or non-pregnant. AL-Faisal *et al.* (2015) showed increased Frequency of micronuclei (MN) in women with RSA caused by genomic instability in somatic cells. These results suggest that MN may be a useful prognostic

**Table 1 :** Frequency of micronuclei in women with recurrent spontaneous abortion and controls group (Mean+SD).

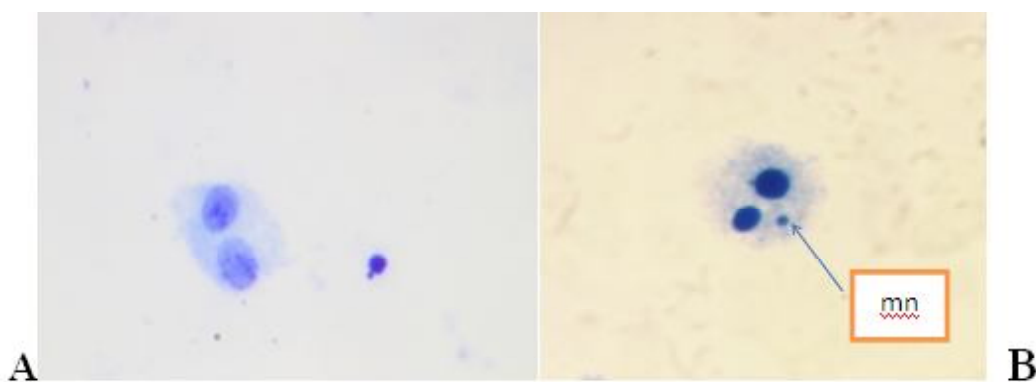
Parameters	Mean ± SD Micronuclei frequency/1000cells			LSD value	P-value
	Group 1 :Control	Group 2:1 abortion	Group:>1 abortion		
Micronuclei frequency-MF	0.0063 ±0.0005 a	0.0041 ±0.0005 b	0.0069 ±0.0009 a	0.0021 *	0.0293

NS: Non-significant. (\*) significant ( $P < 0.05$ ). Different letters: significant difference between means.

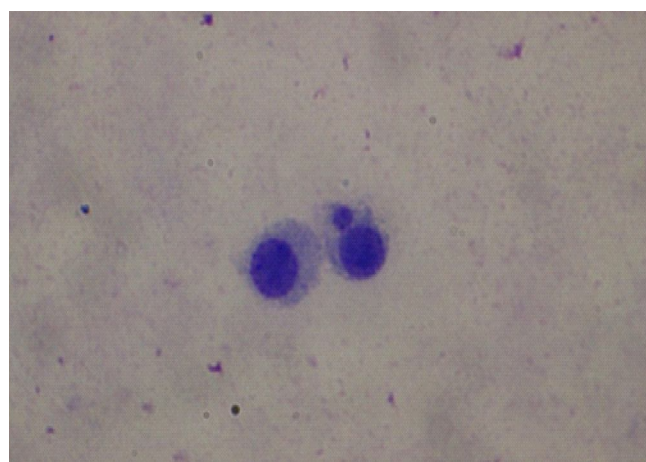
**Table 2 :** Nuclear division index in women with Recurrent spontaneous abortion and controls (Mean±SD).

Parameters	Mean ± SD (%)			LSD value	P-value
	Group 1 :Control	Group 2:1 abortion	Group:>1 abortion		
Nuclear Division Index-NDI	1.583 ± 0.11	1.591 ± 0.02	1.536 ± 0.02	0.059 NS	0.129

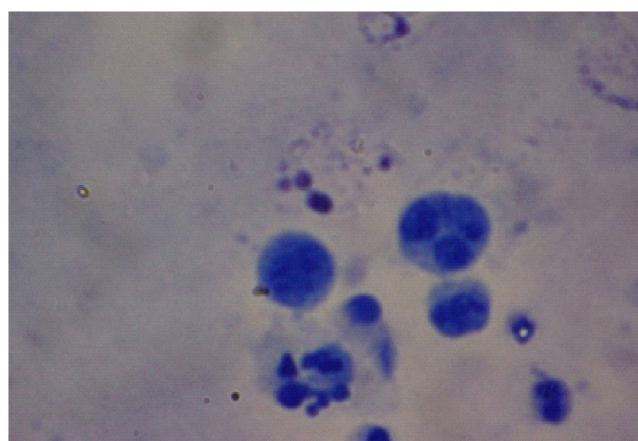
NS: Non-significant.



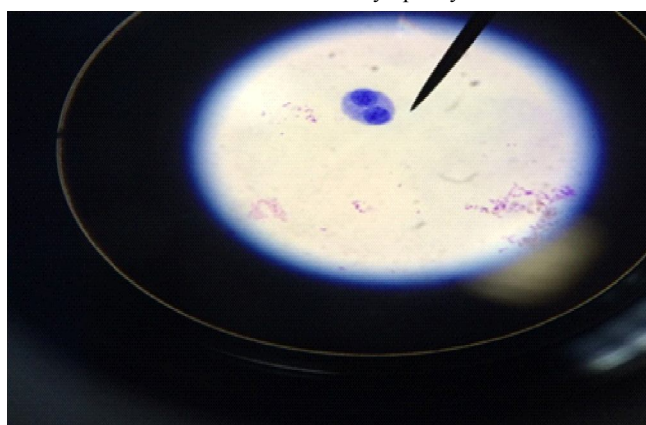
**Fig. 1 :** Bi-nucleated lymphocyte cell of women suffered from spontaneous abortion, without micronucleus (A) and with one micronucleus (B) (100X).



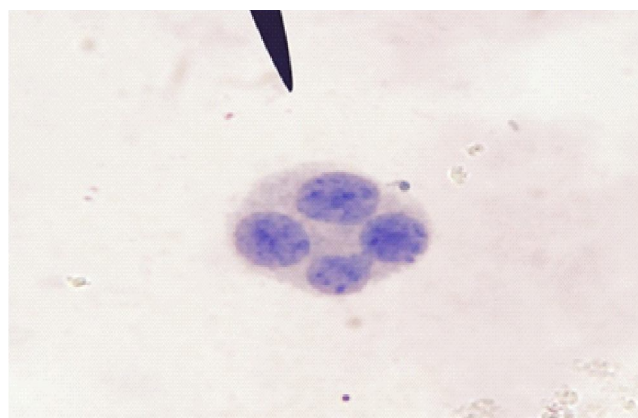
A- mononucleated lymphocyte



C- trinucleated lymphocyte



B- binucleated lymphocyte



D- tetranucleated lymphocyte

**Fig. 2 :** Cytokinesis blocked human lymphocyte cell, (A) mononucleated lymphocyte; (B) binucleated lymphocyte; (C) trinucleated lymphocyte; (D) tetranucleated lymphocyte (100X). In women of 22 years suffering from 2 Spontaneous abortion.

marker of a successful pregnancy, as they may provide an index of the genomic instability of the parental and/or fetal tissues. This can be due to inherited factors predisposing genomic instability, nutritional inadequacies, or environmental exposure to genotoxic factors causing genome damage.

The NDI assay was performed according to the description by Eastmond *et al.* (1989) a biomarker of cell proliferation in cultures, is considered a measure of general cytotoxicity. The relative frequencies of the cells may be used to define cell cycles progression of the lymphocyte after mitogenic stimulation and it is affected by the exposure. The NDI was calculated binucleated,

trinucleated and quadrinucleated lymphocyte cell per 1000 lymphocytes. NDI in this study did not show any effect in women with RSA.

AL-Faisal *et al.* (2015) presented evidence that there are significant increases in cell proliferation in Women with RSA, while (Bukvic *et al.*, 2000) presented evidence that shows significant decreases in cell proliferation.

Many studies showed significant differences between the first trimester and second trimester of the women with abortions group with P value ( $P < 0.05$ ), and there was significant difference between first trimester and control with P value ( $P < 0.05$ ). There was also a significant difference between the second trimester and control groups with P value ( $P < 0.05$ ) (Hindi, 2012; Mahood, 2015).

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