

Cytogenetic and hematological study for some Iraqi women suffer from abortion

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Abstract:

Comet assay (Single cell electrophoresis assay ,SCGE) is a very sensitive method 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). Fifteen blood samples were collected from women complaining with RSA and 5 normal fertile females, who had at least one or more child. The results showed that patients female with RSA had a significant higher DNA damage than in the control group.

Keyword: Comet assay, Recurrent spontaneous abortion.

Introduction:

Recurrent spontaneous abortion (RSA) is a natural termination of two or more successive pregnancies before the fetus is capable of external life (20th weeks) (1). There are numerous factors that might be associated with RSA , but the underlying problem often remains undetected (2 ,3). The known causes of RSA including chromosomal abnormalities, uterine and placental anomalies , hereditary thrombophilia , hormonal problems, nutritional and environmental factors, infection, and immunologic factors (4,5).

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 (6).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- α) (7). As well As, the hormonal cause; the study of which suggested that abnormal levels of one or more hormones might help in forecast RSA (8). 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

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(9). 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 (10). 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 with advanced age and these abnormalities include translocation and deletion (11).

Genetic causes is common in recurrent spontaneous abortions (12) . About half of all early abortion occurs due to a genetic problem within the ova or sperm (13). Most of the pregnancy losses are caused by chromosomal aberrations in the fetus (14,15). Approximately 50% to 60% of all pregnancy losses, caused by chromosomal aberrations (16) . Cytogenetic analysis is necessary in each case to confirm the diagnosis and to assess genetic implication for the family (17). The cytogenetic analyses should be also recommended in couples with RSA (15) because the cytogenetic results could provide important information for their genetic counseling and future genetic prevention (18).

Human biomonitoring studies using the Comet assay provide an efficient tool for measuring human exposure to genotoxicants, thus helping in risk assessment and hazard identification. The assay has been widely used in studying DNA damage and repair in healthy individuals (19). Human biomonitoring using the Comet assay is advantageous since it is rapid, cost effective, with easy compilation of

data and concordance with cytogenetic assays (20). There are two methods of quantification by using image analysis software comet score. The analysis software will calculate different parameters for each comet and three parameters are estimated to indicate DNA migration. Tail length {distance from the head center to the end of the tail} also refers to tail moment {product of tail DNA\total DNA by the tail center of gravity} and tail DNA%=100X Tail DNA Intensity/Cell DNA Intensity (26). These parameters have been widely used by many workers for genotoxic studies(27). Comet assay is useful technique to determine DNA damage which may be cause RSA (21,22), DNA Fragmentation in Male Couples of females with RSA (23,10).

Although many factors associated with SRA are studied in Iraqi women even chromosomal changes as mentioned above. Therefore this project has been proposed to do that.

Materials and Methods:

■ Subjects and Selection of the patients.

Fifteen women were used in this experiment, divided into two groups, also 5 blood samples from normal delivery women with an age range from (20 – 45) year. as the following:

- ▶ Group (I) is normal delivered pregnancy (n=5).
- ▶ Group (II) represents aborted women for the first time during the first and second trimester (n=6).
- ▶ Group (III) represents repeated aborted women during the first trimester (n=9).

■ Blood sample for comet assay

Two ml from venous blood was collected by venipuncture using a 5ml disposable syringe, then drawn into heparinized tube for DNA damage assessment by using Alkaline comet assay. The lymphocytes are isolated from the heparinized whole blood, 2 ml of blood is centrifuged at 1000 rpm for 15 min. The buffy coat was collected in the test tubes and diluted with phosphate buffered saline PBS (cell suspension). The same amount of blood was collected from the control group (5samples). Comet assay is used to determine DNA damage. Comet assay kit is used to perform the test (24).

■ Preparation of Samples and Slides

▶ Oxiselect comet agarose is heated to 90-95°C in a water bath for 20min until agarose liquefies then transferred into 37°C water bath for 20 min.

▶ Cells suspension is centrifuged at 1500 rpm for 2 min. The

supernatant is discarded and the pellet washed once with ice cold PBS (without Mg²⁺ and Ca²⁺) and centrifuged at 1500 rpm for 2 min. then supernatant was discarded.

▶ Cell sample is combined with comet agarose at 1:10 ratio (V/V) and the mixture (75µl/ well) immediately is added into slide comet by pipette.

▶ The slides are hold horizontally then transferred to 4°C in a dark container for 15 min.

▶ The slide is transferred to a small basin containing pre

chilled lysis buffer (25ml/slide), the slide was immersed in the buffer for 30-60min at 4°C in a dark container.

▶ The lysis buffer is aspirated from the container and replaced with pre- chilled alkaline solution (25ml/slide). The slide is immersed in the solution for 30 min at 4°C in the dark container.

▶ The alkaline solution is aspirated from the container and replaced with pre-chilled TBE electrophoresis solution. The slide is immersed for 5min (This step is repeated time).

▶ The slide is hold horizontally, then transferred to a horizontal electrophoresis chamber filled with a cold TBE electrophoresis solution, 1volt/cm voltage is applied to the chamber for 10-15 min.

▶ The slide is hold horizontally then transferred from the electrophoresis chamber into small basin containing per chilled distilled water (25ml/slide). The slide is immersed for 2min (The step repeated twice).

▶ The slide is transferred into container containing 70% ethanol for 5 min then air dried.

▶ Diluted vista green DNA dye 100 µl is added to each well comet assay slide and incubated at room temperature for 15 min.

▶ The slide is examined by fluorescence microscopy using a FITC filter green. Photos were taken for each slides (it was selected 50 random cell was calculated for each sample).

▶ The score for ratio between DNA body and DNA tail was taken by using a specific Microsoft program (Image analysis software comet score).

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage and Least significant difference –LSD test was used to significant compare between means. Correlation coefficient estimate between parameters of comet assay in this study.

Results and Discussion:

Results of the comet assay summarized in the table (1) when comparing between different groups in tail length are highly significant (P<0.01) in group 2 (5.692 ± 0.14) compared with group 1 (0.805 ± 0.07) and with control (0.644 ± 0.05). But there was non-significant change between group 1 (0.805 ± 0.07) compared with control (0.644 ± 0.05).

Tail DNA shows highly significant (P<0.01) in group 2 (20.462 ± 1.92) compared to control (1.542 ± 0.08) and with groups 1 (1.373 ± 0.17). There is non-significant change between group 1 (1.373 ± 0.17) and the control group (1.542 ± 0.08). Tail mean shows highly significant (P<0.01) in group 2 (1.084 ± 0.03) compared with control (0.015 ± 0.002) and with groups 1 (0.029 ± 0.01). So there is a non-significant change between group 1 (0.029 ± 0.01) and control group (0.015 ± 0.002).

Table 1: Parameters of comet assay in women with RSA and control groups (mean + SD).

The Group	Mean ± SD		
	Tail length	Tail DNA	Tail mean moment
Control	0.644 ± 0.05 a	1.542 ± 0.08 a	0.015 ± 0.002 a
Group I	0.805 ± 0.07 a	1.373 ± 0.17 a	0.029 ± 0.01 a
Group II	5.692 ± 0.14 b	20.462 ± 1.92 b	1.084 ± 0.03 b
LSD value	0.327 **	4.272 **	0.067 **
P-value	0.0001	0.0001	0.0001

(**) highly significant decrease (P<0.01).

(a ,b) represent the significant difference between groups for parameters of comet assay in women with RSA and controls.

In table (2) when comparing between control groups and patients in tail length there is a highly significant change (P<0.01) between patients (3.708 ± 0.63) compared to controls (0.644 ± 0.05). This is while tail DNA illustrated

a significant increase (P<0.01) between patients (10.47 ± 3.09) compared to controls (1.542 ± 0.08). While tail mean moment shows non-significant change between patients (0.524 ± 0.16) compared to controls (0.015 ± 0.002).

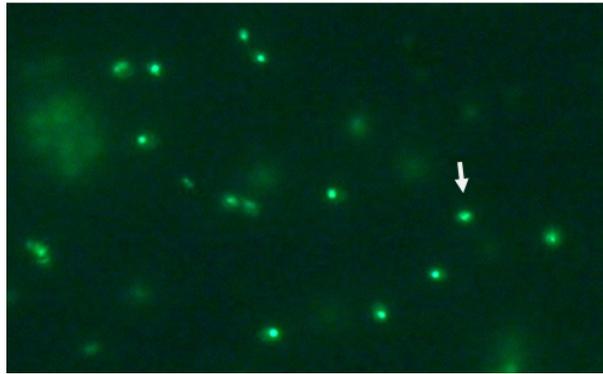
Table 2: Compare between patients and control in comet assay parameters.

The Group	Mean ± SD		
	Tail length	Tail DNA	Tail mean moment
Control	0.644 ± 0.05 a	1.542 ± 0.08 a	0.015 ± 0.002 a
Patients	3.708 ± 0.63 b	10.47 ± 3.09 b	0.524 ± 0.16 a
LSD value	2.364 **	8.047 *	0.497 NS
P-value	0.013	0.0419	0.104

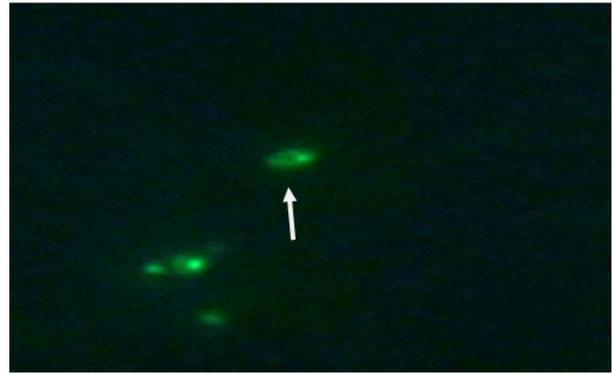
(*) significant decrease (P<0.05).

(**) highly significant decrease (P<0.01).

(a ,b) represent the significant difference between groups for parameters of comet assay in patients and controls.



(A)



(B)

Figure 4.3 : Photomicrograph of fluorescent microscope of comet analysis. DNA of peripheral blood lymphocytes after comet assay (A): DNA without damage. (B) DNA with severe damage , in women 38 years suffering from 4 Spontaneous abortion (40X).

The comet assay was based on the ability of negatively charged fragments of DNA to be drawn through an agarose gel in response to an electric field. The extent of DNA migration depends directly on the DNA damage present in the cells (25).

In this study, DNA strand breakage is examined by comet assay in peripheral blood lymphocytes. The results demonstrate a high significant increased in DNA strands breakage in women with RSA compared to controls. These significant differences may be explained as results of the mothers exposure to radiological materials, chemicals, infection or high temperature, these factors may lead to breakage of DNA strand and subsequently to loss of a piece or the complete gene. Baltaci et al., 1998 (8) showed there was a significant difference in the number of damaged cells. The cells are evaluated according to their grades of damage as: normal (undamaged-no migration), limited migration, (at low damage level) and extensive migration (comet imaged cells-with increasing numbers of breaks, DNA pieces migrate freely into the tail forming a comet image)(21).

The results of comet assay indicate that tail length, DNA and moment were significantly ($p \leq 0.05$) high in RSA women in comparison with its counterpart control group (22).

Comet assay (Single cell electrophoresis assay ,SCGE) is a very sensitive method to determine level of DNA damage, at least in lymphocyte, caused by endogenous (aging, cancer, chronic disease, reactive oxygen species ROS) and exogenous (occupational exposure, smoking-drinking habits, radiation exposure) that affect infertility. These parameters need to be considered in each biomonitoring study. Other studies consider that females are more sensitive against the DNA damage caused the smoking (28).

The comet assay test are fast and effective tools for assessing the primary toxic effects induced by chemical and physical agents (29). In this study, because of the highly significant differences appeared in Comet assay and since its rapidity, low cost effectiveness, the assay compilation of data and its concordance with cytogenetic assays. So it is considered as the best of any method for detecting DNA damage at the level of the individual cell.

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دراسة وراثية خلوية ودمية لبعض النساء العراقيات اللاتي يعانين من الاسقاط

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الخلاصة:

تعد تحليل المذنب (تحليل الترحيل الكهربائي لخلية مفردة) طريقة حساسة جدا لتحديد الدنا المتكسر بسبب التعرض للمواد المسرطنة والعوامل البيئية الاخرى والتي تؤثر على الخصوبة. كان الهدف من هذه الدراسة تقييم احتماليه عدم الاستقرار الجيني للنساء اللاتي يعانين من الاسقاط التلقائي المتكرر. شملت الدراسة 15 عينة دم من نساء اللاتي يعانين من الاسقاط التلقائي المتكرر و 5 عينات لنساء طبيعيات. حيث اظهرت النتائج وجود ارتفاع معنوي في نسبة الدنا المتكسر في مجموعة الاجهاض التلقائي مقارنة مع مجموعة السيطرة .