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DNA Fragmentation in Male Couples of Females with Recurrent Spontaneous Abortions (RSA)

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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 and carcinogenic and environmental agents that effect couples infertility. The aim of project was to evaluate DNA damage in males couples of females with recurrent spontaneous abortions. Forty males couples of females with unexplained recurrent spontaneous abortions and 25 healthy fertile males (control group) were evaluated for semen quality by conventional semen parameters, and alkaline comet assay was applied for the two groups. The results showed that patients male couples of females with RSA had a significant higher DNA damage than in the control group. It was concluded that patients male couples of females with RSA had high score of comet assay which suffers from RSA.

Keywords: DNA fragmentation, males, Recurrent Spontaneous Abortions.

تكسر الدنا في ذكور الزوجات اللاتي تعاني من الاجهاض التلقائي المتكرر الغير معروفة الاسباب

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الخلاصة

تعد تحليل المذنب (تحليل الترحيل الكهربائي لخلية منفردة) طريقة حساسة جدا لتحديد الدنا المتكسر بسبب التعرض للمواد المسرطنة والعوامل البيئية الاخرى والتي تؤثر على الخصوبة. كان الهدف من هذه الدراسة تقييم الدنا المتكسر في ذكور الزوجات اللاتي عانين من الاجهاض التلقائي المتكرر. شملت الدراسة 40 ذكرا لزوجات عانين من الاجهاض التلقائي المتكرر و 25 ذكرا اصحاء وخصيين كمجموعة سيطرة، وتم تقييم السائل المنوي لهم باستخدام الطريقة الاعتيادية لتقييم السائل المنوي. وتم استخدام طريقة تحليل المذنب القاعدية لجميع المجاميع، حيث اظهرت النتائج وجود ارتفاع معنوي في نسبة الدنا المتكسر في مجموعة الاجهاض التلقائي المتكرر مقارنة مع مجموعة السيطرة. واستنتج من نتائج هذه بان نسبة تحطم الدنا لدى ذكور الزوجات اللاتي عانين من الاجهاض التلقائي المتكرر اعلى من الطبيعيين.

Introduction:

Recurrent spontaneous abortion (RSA) is defined as three or more consecutive losses of pregnancy [1], and the etiology in about 60 % of cases is not known [2]. The main etiology that causes RSA may include, chromosomal, anatomical, hormonal, immunological and other still unknown factors [3]. Male factor is also considered in the etiology of RSA. Sperm motility, viability and function could lead to RSA [4]. The increase in spontaneous abortion rate was observed in patients with DNA fragmented sperm [5]. DNA damage in the male germ line is associated with fertilization failure, impaired pre- and post- implantation development and poor pregnancy outcome. Reduction in the sperm function in couples with idiopathic RSA may lead to formation of unsustainable embryo and result in early pregnancy loss [4]. It has been shown that sperm with head abnormalities, defects of

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chromatin condensation and irregular nuclei with vacuoles are likely to be as possible male factors that contribute to early pregnancy loss [6].

The relationship between sperm DNA damage and pregnancy loss is unclear [7]. However, it has been revealed that sperm with damaged DNA can fertilize the oocyte at *in vitro* fertilization (IVF) successfully [8] and has no effect on normal development of embryo [9]. The insignificant increase in spontaneous abortion (RSA) rate was observed in patients with DNA fragmented sperm [5]. Therefore, the integrity of the sperm DNA is important for having healthy offspring.

In some cases it has been found that sperm DNA damage correlates with infertility, elevated risk of early pregnancy loss, defective embryogenesis, major and minor congenital malformations, genetic and epigenetic abnormality and prenatal morbidity [10,11]. The objective of this study to determine the effect of DNA damage measured by alkaline comet assay on partner idiopathic RSA.

Materials and methods

Study design:

Patients were selected according to clinical and laboratory examination. Forty male couples of females with RSA and 25 fertile men, at least having one child, were examined as the control group. They were evaluated for semen quality by conventional semen parameters for patient's male couples of females with RSA and infertile men with normal semen profile.

Semen analysis:

According to [12,13].The semen analysis test is usually done manually.

Drops of semen were placed on a slide and examined under the microscope. Motility, or movement, of 100 sperm were observed and graded in categories, such as rapid progressive or immotile.

Sperm morphology was assessed by carefully examining sperm for abnormalities in the size and shape of the head, tail, and neck regions. World Healthy Organization (WHO) [14] standards define normal as a specimen with less than 30% abnormal forms. An alternative classification system measures the dimensions of sperm parts. Normal specimens are allowed 14% or less abnormalities WHO [14].

Blood sample:

The study was carried out in the laboratories of Biotechnology Research Center, AL-Nahrain University, Baghdad, from March till July 2015. Five mL of blood was collected by vein puncture obtained from some Baghdad Hospitals and physicians private. Each collected blood sample was dispensed into tubes with EDTA tubes for comet assay analysis.

Isolation and Counting of lymphocytes:

Lymphocytes were isolated from whole blood sample depending on the method described by Boyum, [15], using Ficoll-isopaque separation fluid (lymphoprep; specific gravity = 1.077).

Counting the lymphocytes cells were performed before experiment according to Porakishvili *et al.*, [16] by using trypan blue stain and counting by hemocytometer, then the viability was determined according to the following equation:

$$\text{Viability (\%)} = \left(\frac{\text{Number of Living Cells}}{\text{Total Number of Cells}} \right) \times 100$$

The cells number was adjusted to 1×10^6 cell/mL.

Alkaline comet assay:

Alkaline comet assay will detect single and double-stranded DNA breaks, and the majority of a basic sites as well as alkali labile DNA adducts (*e.g.* phosphoglycols, phosphotriesters).

1. Prepared lysis Solution and cooled at 4°C for at least 20 minutes before use.
2. Melted agarose low melted (LM) in a beaker of boiling water for 5 minutes, with the cap loosened, and placed in 37°C water bath for at least 20 minutes to cool.
3. Cells were combined at 1×10^5 /mL with molten LM Agarose (at 37°C) at a ratio of 1:10 (v/v) and immediately 50 µl pipetted onto comet slide.
4. Slides flat were placed at 4°C in the dark for 10 minutes.
5. Slides were immersed in 4°C lysis solution for 30-60 minutes.
6. Comet Slide were immersed in alkaline unwinding solution for 20 minutes at room temperature or 1 hour at 4°C, in the dark.
7. Electrophoresis solution was added, slides were placed in electrophoresis slide tray (slide labeled adjacent to black cathode) and covered with slide tray overlay. power supply was setted to 21 volts and voltage was applied for 30 minutes.

8. Excess electrophoresis solution was drained gently immersed twice in dH₂O for 5 minutes each, then in 70% ethanol for 5 minutes.
9. Samples were dried at 37°C for 10-15 minutes.
10. 100 µl of diluted SYBR Green was placed onto each circle of dried agarose and stained 30 minutes (room temperature) in the dark.
11. Slide was gently tapped to removed excess SYBR solution and rinsed briefly in water. Slides were dried completely at 37°C. Slides were viewed by fluorescence microscopy.

Comet assay scoring:

Fifty randomly selected cells were counted per sample to quantify the comet cell. Scored was calculated from the ratio of (L/W) comet to determine the Comet Index (CI). Scored range from 1.2 to 2 considered low DNA damage (LD), from 2.1 to 3 medium DNA damage (MD), and up to 3 high DNA damage (HD) [17, 18].

The way of quantification by using image analysis software comet score. The analysis software will calculate different parameters for each comet. Three parameters were estimated to indicate DNA migration; tail length (distance from the head center to the end of the tail), mean tail moment (product of tail DNA/total DNA by the tail center of gravity) and tail DNA%=100X Tail DNA Intensity/Cell DNA Intensity [19,20]. Figure-1A,B.



Figure 1- Example of scoring and analysis for comet assay by comet score program. A: with high comet B:normal cell

Statistical analysis:

All data were analyzed by using one-way analysis of variance (ANOVA- test) with significant differences ($P \leq 0.05$) and the data were presented as means \pm SD.

Results and Discussion:

The results of patients and control semen analysis are shown in Table-1. Highly score mean comet (%) of lymphocyte in in male couples of females with RSA compared with control group, include: No damage (ND), Low damage (LD), Medium damage (MD) and High damage (HD) are shown in Figures-2 and 3.

Table 1- Semen analysis of patients and controls

Parameters	RSA (Range)	Control (Range)	References range (WHO,2010)
Gross examination			
Volume (ml)	1.7-2.6	1.8-2.9	More than 1.5 ml
pH	7.4-7.7	7.5-7.6	7.2-7.6
Liquefaction (minute)	30-34	32-35	Complete in 30 min
Microscopic examination			
Count (million/ml)	18-32	24-35	More than 15 million/ml
Total motility(%)	45-71	52-75	More than 50%

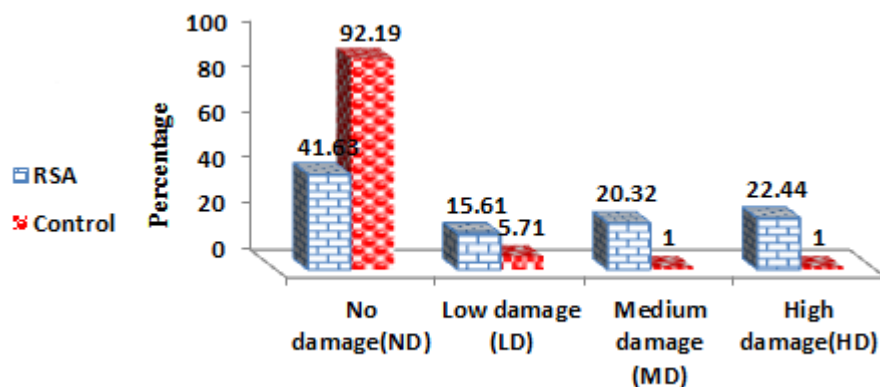


Figure 2- Score mean comet (%) of lymphocyte in males couples of females with RSA and control groups

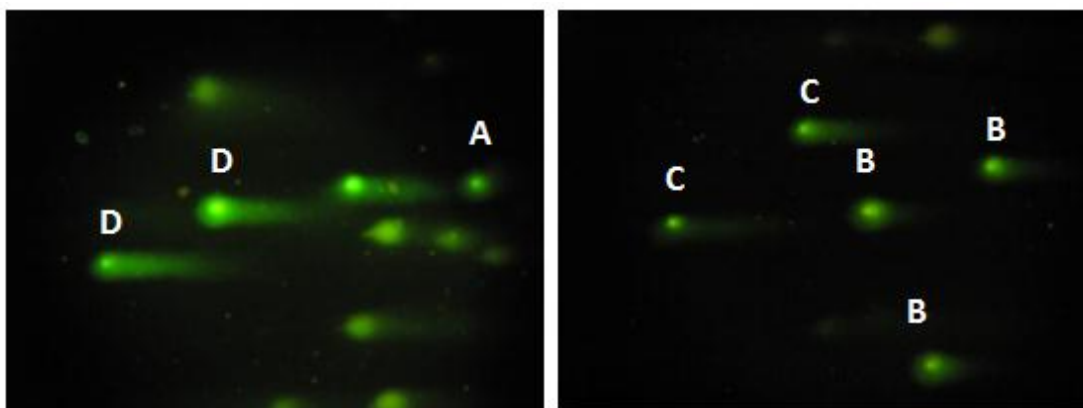


Figure 3- Three examples of scoring categories for comet assay (A: Normal; B: Low DNA Damage (LD); C: Medium DNA Damage (MD); D: High DNA Damage (H)) in patients lymphocytes cells.

The results also showed significant increase ($P < 0.05$) in comet assay percentage in male couples of females with RSA compared with control group tail length (9.22 ± 0.95 vs. $0.83 \pm 0.059\%$), tail DNA (44.51 ± 6.28 vs. $2.36 \pm 0.74\%$) and tail mean moment (3.26 ± 0.48 ; $0.088 \pm 0.007\%$) Table -2.

Table 2- A mean \pm SD for comet assay percentage in patients and control groups

Groups	Mean \pm SD		
	Tail length(px)pixels	Tail DNA (%)	Tail mean moment
Male couples of females with RSA	9.22 ± 0.95	44.51 ± 6.28	3.26 ± 0.48
Control	0.83 ± 0.059	2.36 ± 0.74	0.088 ± 0.007
$P \leq$	0.05	0.05	0.05

There are no significant differences in semen analysis in male couples of females with RSA and healthy control group. Conventional semen analysis to detected sperm abnormalities are not reliable indicator for semen quality [21].

The study showed that the male couples for females with recurrent spontaneous abortion have highly frequency of DNA fragmentation more than normal males, this results agreement with many previous studies [21- 23]. Male factor considered one of important etiology caused RSA [24], the increase of frequency DNA fragmentation in males effect on embryogenesis and implantation through effected on head abnormalities, defect on chromatin condensing and irregular nucleolus which lead to RSA/early stage of pregnancy [1,2]. Comet assay is a very useful technique in assessing DNA damage and is an important prerequisite in men opting for assisted reproduction technique (ART), in couples experiencing recurrent ART failure and in cases of bad obstetric history. However for technical and biological reasons, the comet assay underestimates the true frequency of DNA breaks. This may be due to several possible causes: [23,25], masking, overlapping and entangling of migrating fragments,

incomplete chromatin de-condensation may not allow all breaks to be revealed and due to loss of small pieces of DNA from agarose during various steps involved in the comet assay there may be fragments which are too small to be visualized.

Thus the DNA damage observed is less than the actual DNA damage providing an approximate assessment for level of DNA damage. However, since significant effect of DNA damage is only observed when DNA fragmentation index (DFI) is greater than 27 to 30 per cent, this technique proves to be ideal in diagnostic workup of idiopathic infertile men and couples with bad obstetric history [26].

In conclusion normal structure of sperm chromatins not important only for fertilization ability but had important role in implantation and embryogenesis. Comet assay useful technique to determined DNA damage which may be cause recurrent abortion from male factor.

References:

1. Stirrat, G.M.1990. Recurrent pregnancy loss I: definition and epidemiology. *Lancet*; 336, pp: 673-675.
2. Coulam, C.B.1991. Epidemiology of recurrent spontaneous abortion. *Am J. Reprod Immunol*; 26,pp: 23-27.
3. Gupta, S., Agarwal, A., Banerjee, J., and Alvarez, J.G.2007. The role of oxidative stress in spontaneous abortion and recurrent pregnancy loss: A systematic review. *Obstet Gynecol Surv*, 62,pp: 335-347.
4. Saxena, P., Misro, M.M., Chaki, S.P., Chopra, K., Roy, S., and Nandan, D.2008. Is abnormal sperm function an indicator among couples with recurrent pregnancy loss. *Fertil Steril*; 90, pp: 1854-1858.
5. Ming-Huei Lin, Robert Kuo-Kuang Lee, Sheng-Hsiang Li, Chung-Hao Lu, Fang-Ju Sun, and Ming Hwu.2008. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. *Fertil Steril*; 90,pp: 352-359.
6. Gopalkrishnan, K., Padwal, V., Meherji, P.K., Gokral, J.S., Shah, R., and Juneja, H.S.2000. Poor quality of sperm as it affects repeated early pregnancy loss. *Arch Androl*, 45,pp: 111-117.
7. Zini, A., Boman, J.M., Belzile, E., and Ciampi, A.2008. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. *Hum Reprod*, 23,pp: 2663-2668.
8. Gandini, L., Lombardo, F., Paoli, D., Caruso, F., Eleuteri, P., and Leter, G.2004. Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. *Hum Reprod*, 19,pp: 1409-1417.
9. Bungum, M., Humaidan, P., Spano, M., Jepson, K., Bungum, L., and Giwercman, A. 2004.The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod*, 19,pp: 1401-1408.
10. Moskovtsev, S.I., Willis, J., White, J., and Mullen, J.B. 2009. Sperm DNA damage: correlation to severity of semen abnormalities. *Urology*, 74, pp:789-93.
11. Zini, A., and Sigman, M.2009. Are tests of sperm DNA damage clinically useful? Pros and cons. *J. Androl.*,30, pp:219-29.
12. Hiniting, A.1989. Methods of semen analysis in and Assessment of human sperm fertilizing ability. Ph.D. Thesis, Mishigan University.
13. Rupert, A. and Dagmar, W. 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology*, 81,pp:5-17.
14. World Health Organization.2000. WHO Manual for the Standardised Investigation and Diagnosis of the Infertile Couple. Cambridge: Cambridge University Press.
15. Boyum, A.1968. Separation of lymphocyte from blood and bone marrow. *Scand .J.clin .lab.invent* .21(suppl .97),pp:77-89.
16. Porakishvili,V., Kardava, L., Jewell, A., Yang, K., Glennie, M., Akbar, A. and Lydyard, P.2004. Cytotoxic CD4 Tcells in patients with Bcell chronic lymphocytic leukemia kill via aperforin mediated pathway. *Haematological*. 89,pp: 435-443.
17. Collins,A.R., Harrington, V., Drew, J. and Melvin, R. 2003. Nutritional modulation of DNA repairs in a human intervention study. *Carcinogenesis*. 24,pp: 511-515.

18. Al-Jewari, H.S.J. **2010**. *In vitro* Cytotoxic Activity of the L-asparaginase Extracted and Purified from Pathogenic *Escherichia coli* against four Leukemic cell lines. Ph.D. Thesis, Genetic Engineering Biotechnology for Post Graduate Studies, University of Baghdad, Iraq.
19. Gontijio, A., Elias, F., Salvadori, D., Oliveira, M., Correa, L. and Goldberg, J. **2001**. Single cell gel comet assay detects primary DNA damage in nonneoplastic urothelial cells of smokers and Ex smokers. *Cancer Epidemiology biomarkers and prevention*, 10, pp:987-993.
20. Azqueta, A., Shaposhnikov, A. and Collins, A. **2009**. Mutation research genetic toxicology and environmental mutagenesis. *Mutation Research.*, 674,pp:101-108.
21. Shamsi, S., Venkatesh, M., Tanwar, G., Singh, S., Mukherjee, N., Malhotra, R., Kumar, N., Gupta, S. and Mittal R. **2010**. Comet assay: A prognostic tool for DNA integrity assessment in infertile men opting for assisted reproduction. *Indian J Med Res*, 131, pp: 675-681
22. Fernandez, J.L., Mouriél, L., Rivero, M.T., Goyanes, V., Vasquez, R. and Alvarez, J.L. **2003**. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. *J. Androl.*, 24,pp: 59-66.
23. Dada, R. **2009**. Recurrent pregnancy loss: Male factor. In: Deka D, 28. Malhotra N, editors. *An introduction to genetics and fetal medicine. New Delhi: Jaypee Publications*, pp: 31-37.
24. Shamsi, M.B., Kumar, R., Bhatt, A., Bamezai, R.N.K., Kumar, R., and Gupta, N.P. **2008**. Mitochondrial DNA mutations in etiopathogenesis of male infertility. *Indian J. Urol*, 24, pp: 150-154.
25. Olive, P.L., Wlodek, D., Durand, R.E., and Banath, J.P. **1992**. Factors 29. influencing DNA migration from individual cells subjected to gel electrophoresis. *Exp Cell Res*, 198, pp: 259-267.
26. Evenson, D.P., Larson, K.L. and Jost, L.K. **2002**. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with the other techniques. *J. Androl.*, 23,pp: 25-43.