CLINICAL STUDY

Increased micronucleus frequencies in peripheral blood lymphocytes in women with polycystic ovary syndrome

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Abstract

Objective: We aimed to assess possible genomic instability in women with polycystic ovary syndrome (PCOS).

Design: The frequency of micronuclei in cultured peripheral lymphocytes was used as a biomarker of genomic instability in somatic cells.

Methods: Nineteen women, diagnosed with PCOS and 19 healthy female volunteers of corresponding ages and body-mass index (BMI) were included in the study. Micronuclei frequencies were assessed in cytokinesis-blocked lymphocytes.

Results: The frequency of micronucleated cells (per thousand) was 9.00 (5.00) (interquartile range in parentheses) for patient group and 3.0 (3.0) for the control group ($P < 0.0001$, Mann-Whitney U-test). The serum levels of follicle-stimulating hormone (FSH), estradiol, prolactin, glucose and dehydroepiandrosterone sulfate (DHEAS) and the homeostasis model of assessment of insulin resistance (HOMA-IR) were not different between the two groups ($P > 0.05$). Serum total testosterone, luteinizing hormone (LH) and insulin levels and hirsutism score in the PCOS group were significantly ($P = 0.007$, $P < 0.0001$, $P = 0.009$ and $P < 0.0001$ respectively) higher than those of the control group (2.3 (2.1) nmol/l vs 1.7 (0.4) nmol/l; 8.5 (5.88) mU/ml vs 4.8 (4.4) mU/ml; 6.8 (5.1) μ U/ml vs 9.7 (4.2) μ U/ml; 19.5 (6.5) vs 4.0 (2.5) respectively). However, the mean level of sex hormone-binding globulin (SHBG) in PCOS group was significantly ($P = 0.004$) lower than in control group (36.4(22.6) nmol/l vs 48.6(25.2) nmol/l respectively).

Conclusion: These findings suggest that women with PCOS have a high incidence of genomic instability, and this condition is positively correlated with the hirsutism score, BMI, LH and serum total testosterone and insulin levels, and is negatively correlated with SHBG.

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Introduction

Polycystic ovary syndrome (PCOS), characterized by hyperandrogenism and chronic anovulation, is a common endocrine disorder in women. PCOS, which is associated with polycystic ovaries, hirsutism, obesity and insulin resistance, is a leading cause of female infertility (1). Recently, it has been suggested that women with PCOS show higher levels of leukocyte count, a marker of low-grade inflammation and cardiovascular risk, than controls (2). Epidemiologic studies have demonstrated that the prevalence of PCOS in women is 3.5–10%, depending on the diagnosis criterion (3, 4).

The genetic mechanisms underlying PCOS remain largely unknown (5). Although an autosomal dominant model was proposed (6), later studies did not confirm

this. Chromosomal studies in patients with PCOS have produced contradictory findings. There have been reports that X-chromosome aneuploidies and polyploidies (7), as well as other cytogenetic abnormalities (8), are associated with PCOS in a limited number of subjects. One well-characterized case had both endocrine and ultrasound stigmata of PCOS (as well as multiple other anomalies, including heart and facial dysmorphies) and a large deletion on the long arm of the chromosome 11 (9). However, other cytogenetic analyses have failed to identify common karyotypic abnormalities (10).

A number of studies reported that PCOS is an oligogenic disorder, and more studies are necessary to define its genetic basis (5, 11). Different combinations of multiple gene polymorphisms and environmental factors explain the heterogeneity of PCOS (5).

This study aimed to assess, by cytokinesis-blocked micronucleus (CBMN) assay, possible chromosomal instability in women with PCOS. The micronucleus (MN) frequencies in cultured peripheral blood lymphocytes have been used as biomarkers of chromosome damages for many years. MN are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind at the anaphase stage of cell division. Their presence in cells reflects structural and/or numerical chromosomal aberrations arising during mitosis (12–15). The quantification of MN for the evaluation of chromosome damage offers several advantages: the method is simple and fast, and it does not require the presence of metaphasic cells. Another advantage of the MN assay is the relative ease of scoring and the statistical power obtained from scoring larger numbers of cells than are typically used for metaphase analysis (14). Fenech and Morley (12) presented a CBMN assay that resolved earlier problems experienced with differences in cell growth by monitoring cell division with the aid of cytochalasin B. This agent allows division of the cell nucleus (karyokinesis), but prevents cell division (cytogenesis), thus resulting in binucleated cells (12–14).

Materials and methods

The study comprised 19 women newly diagnosed with PCOS, admitted to endocrinology and metabolism clinics, and 19 control subjects with similar age and body-mass index (BMI). The procedures used were in accordance with the guidelines of the Helsinki Declaration on human experimentation. The study protocol was approved by the ethics committee of Inonu University (Faculty of Medicine). All the human subjects were fully informed and gave written, informed consent.

PCOS was diagnosed by the criteria revised in 2003 (16). Patients who had two or more of the following criteria were defined as having PCOS:

1. history of oligo- and/or anovulation in reproductive years (<6 menses per year, not in the last 2 years prior to menopause)
2. clinical and/or biochemical signs of hyperandrogenism: hirsutism score of >6 and/or high total testosterone level
3. typical ovarian histopathology of polycystic ovaries on ultrasound: multiple follicles in each ovary measuring 2–9 mm in diameter and/or increased ovarian volume (>10 ml).

Other causes of hyperandrogenism, such as hyperprolactinemia, Cushing's syndrome, androgen-secreting tumors and congenital adrenal hyperplasia, were excluded by past medical history, physical examination and specific laboratory analysis. Moreover, 17-OH progesterone and 17-OH progesterone responses to

adrenocorticotrophic hormone (ACTH) stimulation test were evaluated in some suspected cases to exclude congenital adrenal hyperplasia.

Hirsutism score was evaluated by a single examiner (dermatologist) by the Ferriman–Gallwey score (FGS), as modified by Hatch *et al.* (17).

Blood samples were drawn during the midfollicular phase (days 3–7) of the menstrual cycle after overnight fasting. Serum total testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, dehydroepiandrosterone sulfate (DHEAS), prolactin, sex-hormone binding globulin (SHBG) and insulin levels were measured by an automated chemiluminescence system (Immulite 2000; Diagnostic Product Cooperation, Los Angeles, CA, USA). Insulin resistance was calculated by the homeostasis method of assessment of insulin resistance (HOMA-IR) (18).

Micronucleus assay

Peripheral blood samples were collected by a heparinized sterile injector. Whole blood (0.5 ml) was added to 5 ml RPMI-1640 medium (Sigma, R0883) with 25% fetal bovine serum (Sigma, F7525), 1% L-glutamine (Sigma, G7513) and 2% phytohemagglutinin (Biological Industries, Kibbutz Beit Haemek, Israel). CBMN assay was carried out by the method of Fenech (13). Cytochalasin B (Serva, 18015, Heidelberg, Germany) was added to the cultures at a final concentration of 6 µg/ml after 44-h incubation stimulation with phytohemagglutinin (14). Cells were harvested after 72-h incubation, and they were treated with a hypotonic solution (0.075 M KCl) for 1 min and fixed in fresh fixative solution (methanol: acetic acid, 3:1). Slides were air-dried and stained with Giemsa. Micronucleated cells were analyzed under light-microscopy ($\times 400$) by scoring 1000 binucleated lymphocytes, in which the number of MN was recorded by standard recognition criteria (13). Briefly, MN were 1. morphologically identical to the main nuclei but their diameters were from 1/16 to 1/3 of the mean diameter of the main nuclei; 2. nonrefractile; 3. not linked to the main nuclei.

Subjects with possible confounding factors (such as exposure to physical and chemical mutagens, history of alcohol and coffee consumption or smoking, medication, viral infections suffered in the last 3 months, vaccination, hereditary diseases) that potentially play a role in the induction or expression of MN (7–9) was not included in the study.

Statistical analysis

Statistical analyses were performed with SPSS for Windows, Version 13.0 (SPSS Inc., Chicago, IL, USA). Data are presented as median and interquartile range. All parameters were tested by the Shapiro–Wilks

normality test, and its distribution was not normal ($P < 0.05$). Therefore, statistical differences between groups were determined with the nonparametric Mann–Whitney U-test. Bivariate correlations were performed by calculating the Spearman coefficient. The level of $P < 0.05$ was considered to be statistically significant (two tails).

Results

The results of MN analysis and biochemical characteristics for healthy controls and women with PCOS are given in Table 1. There were no statistically significant ($P > 0.05$) differences between groups, according to anthropometrics parameters (age, BMI and abdominal circumference). As expected, PCOS patients had significantly ($P < 0.05$) higher hirsutism score. The levels of serum total testosterone, LH and basal insulin were also significantly ($P = 0.007$, $P < 0.001$ and $P = 0.009$ respectively) higher in patients than in controls, whereas the levels of FSH, estradiol, prolactin and glucose were similar ($P > 0.05$) in the two groups. The level of SHBG was significantly ($P = 0.004$) lower than control values. HOMA-IR levels were similar ($P > 0.05$) in patients and controls.

Finally, micronucleated cell rates per 1000 binucleated lymphocytes were significantly ($P < 0.0001$) higher in women with PCOS than in controls. A micronucleated lymphocyte is shown in Fig. 1. There were positive correlations between micronucleated cell rate and hirsutism score ($r = 0.501$, $P = 0.001$), BMI ($r = 0.343$, $P = 0.035$), LH ($r = 0.501$, $P < 0.0001$) and total testosterone ($r = 0.431$, $P = 0.005$). In addition, there was negative correlation between micronucleated cell rate and level of SHBG ($r = -0.397$, $P = 0.014$).

Discussion

In the past decade, the association between hyperandrogenism, hyperinsulinemia and PCOS was demonstrated (19–21). Our findings, summarized in Table 1, are consistent with those of the previous studies, except for HOMA-IR. Although the HOMA-IR score increased in the PCOS group, this difference was not statistically significant ($P = 0.089$), possibly because of the small number of individuals investigated.

One of the most promising methods to assess DNA damage is the CBMN assay, which detects both chromosome and genome alterations in binucleated cells (13). Owing to the direct correlation between MN production and genomic damage, the CBMN assay applied to human blood peripheral lymphocytes may be considered a reliable method to quantify genome-induced chromosome damage and/or genomic instability (12–15). To the best of our knowledge, this is the first study to investigate MN frequencies in lymphocytes of woman with PCOS.

Spontaneous or baseline MN frequencies in cultured human lymphocytes and exfoliated cells provide an index of accumulated genetic damage occurring during the life span of these cells. For the application of the MN test as a cytogenetic marker of chromosomal damage, the background level in the 'normal' population must be specified. Micronucleated cell levels observed in this study averaged 3.0 (3.0) and are consistent with previous findings that micronucleated cell rates in peripheral lymphocytes vary from 1.56 to 11.10 (22–30). The difference between the lower and upper levels in the control group shows that interindividual variability is observed for MN levels in lymphocytes. Interindividual variation may be explained by lifestyle factors, including environmental exposure, or individual susceptibility factors (31).

Table 1 The frequency of MN in peripheral blood and biochemical characteristics for healthy controls and women with PCOS.

	Healthy controls ($n = 19$)	Women with PCOS ($n = 19$)	<i>P</i> value
Age (years)	22.0 (8.0)	23.0 (9.0)	0.491
BMI (kg/m^2)	22.0 (3.0)	23.0 (7.7)	0.081
Abdominal circumference (cm)	74.0 (15.0)	74.0 (12.0)	0.114
Hirsutism score	4.0 (2.5)	19.5 (6.5)	$<0.0001^*$
FSH (mU/ml)	5.4 (4.4)	6.7 (2.1)	0.350
LH (mU/ml)	4.8 (4.4)	8.5 (5.9)	$<0.0001^*$
Total testosterone (nmol/l)	1.7 (0.4)	2.3 (2.1)	0.007*
DHEAS ($\mu\text{g/dl}$)	175 (258)	252 (117)	0.583
Estradiol (pg/ml)	32.3 (22.8)	34.0 (84.0)	0.588
Prolactin (ng/ml)	12.5 (15.6)	13.4 (8.1)	0.919
SHBG (nmol/l)	48.6 (25.2)	36.4 (22.6)	0.004*
Insulin ($\mu\text{U/ml}$)	6.8 (5.1)	9.69 (4.2)	0.009*
HOMA-IR	1.60 (1.2)	1.90 (1.0)	0.089
Glucose (mg/dl)	93.0 (17.0)	91.5 (15.3)	0.890
MN frequency (per 1000 cells)	3.0 (3.0)	9.0 (5.0)	$<0.0001^*$

Values are given as median (interquartile range). * $P < 0.05$ statistically significant (two tails). BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone; DHEAS: dehydroepiandrosterone sulfate; SHBG: sex hormone-binding globulin; HOMA-IR: homeostasis model assessment – insulin resistance; MN: micronucleus.

Figure 1 (A) Binucleated lymphocyte; (B) micronucleated binucleated lymphocyte. 1000X

MN frequencies in peripheral blood lymphocytes were significantly ($P < 0.0001$) higher in women with PCOS than in controls. This result agrees with some of the reports dealing with this issue (7–9, 32). These reports suggest that genetic abnormality is present in women with PCOS. In our study, hyperandrogenism and hyperinsulinemia in PCOS patients might also play a central role in increased MN frequencies in peripheral blood lymphocytes. In translating our results into clinical practice, one must keep in mind that our study population consisted of a relatively small number of patients.

Hando *et al.* (33) showed the X chromosome to be present in 72.2% of the MN that were scored. Although we did not analyze the contents of the MN found in subjects, it has been described that both the X chromosome and autosomes are responsible for the higher rate of MN in peripheral lymphocytes in women (34).

If a subfertile population is reviewed, PCOS is diagnosed in about 75% of patients with anovulatory infertility, and high prevalence rates of PCOS have been reported among women with recurrent miscarriages (35, 36). Balen *et al.* (37) reported a high prevalence of primary (46%) and secondary (26%) infertility in a large group of PCOS patients. In addition, a higher incidence of chromosomal instability in the infertile population is widely recognized (27). Trkova *et al.* (27) showed that MN frequencies are increased in couples with infertility or with two or more spontaneous abortions.

Oxidative stress that arises due to an imbalance between generation of reactive oxygen species (ROS) and antioxidant defense has been linked to a number of disease states, including cardiovascular disease, aging and various cancers (38,39). Similarly, increased oxidative stress and decreased antioxidant capacity were reported in women with PCOS (40, 41). Protein, lipids, RNA and DNA all undergo continual damage by ROS, produced either as a result of normal cellular metabolism or as a result of other types of oxidative stresses. DNA damage is generally in the form of mutation or strand breakages (38). In addition, it was documented that oxidative stress induces chromosomal breakage and formation of bone-marrow MN (39). Furthermore, some authors have shown a positive correlation between the extent of lipid peroxidation and genotoxicity reflected by increased MN formation (42–45).

It is generally accepted that PCOS is associated with an increased risk of cardiovascular disease (41, 46, 47). Orio *et al.* (47) have shown the detrimental effect of PCOS on the cardiovascular system, even in young women asymptomatic for cardiac disease. Another study (28) demonstrated that the frequency of MN in peripheral blood is increased in patients with coronary artery disease.

Women with PCOS are also thought to be at increased risk of cancer (11, 48, 49). It is noteworthy that some investigators have shown that in lymphocytes the MN levels are twofold higher in cancer patients than in corresponding healthy individuals (50, 51). Our finding of increased MN frequency in PCOS may contribute to a better understanding of endogenous mutation process and thus determination of cancer risk in these patients.

In conclusion, this study demonstrated an association between increased micronucleus frequency and PCOS. Our findings suggest that there is genetic instability in peripheral blood lymphocytes in women with PCOS. We suggest that hyperandrogenism, hyperinsulinemia and perhaps oxidative stress are factors contributing to increased MN frequency and chromosomal damage.

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