Cytogenic effects of endogenous sex hormones depending on smoking habits

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Abstract: This study was conducted with the aim of determining the effects of periodical hormonal fluctuations related to the menstrual cycle on chromosome sensitivity and cytotoxicity. The study group consisted of 8 healthy donors (4 nonsmokers and 4 smokers). Cytogenetic tests were done in vitro (in test tubes), and the known mutagenic effect of mitomycin C was added to determine chromosome sensitivity. Blood was drawn from the donors at specified time intervals (follicular phase, ovulation phase, and luteal phase), and control groups and mitomycin C-treated groups were formed. In the controls, the highest sister chromatid exchange frequency was detected in the follicular phase and the lowest frequency was detected in the luteal phase of nonsmokers. In smokers, the highest sister chromatid exchange frequency was detected in the ovulation phase and the lowest frequency was detected in the luteal phase. In terms of chromosomal aberrations, the highest values were detected in the follicular phase and the lowest values were detected in the luteal phase in nonsmokers. On the contrary, the highest rate of anomaly was detected in the luteal phase and the lowest rate of anomaly was detected in the follicular phase in smokers. However, there was no statistically significant difference between these findings. The data of the MMC application were similar in both groups. In this study, both the follicular phase and the ovulation phase showed slightly higher chromosome sensitivity, while the chromosomes in the luteal phase were the most stable. These results are probably due to hormonal fluctuation.

Key words: Menstrual cycles, endogenous sex hormones, chromosome sensitivity, sister chromatid exchange, chromosome aberrations

1. Introduction
Sex hormones (such as estradiol [E2], progesterone, follicle stimulating hormone [FSH], and luteinizing hormone [LH]) periodically fluctuate within certain ranges in healthy adolescents and nonpregnant women of reproductive age. The menstrual cycle is divided into 3 phases (follicular phase, ovulation phase, and luteal phase) when this fluctuation is taken into consideration. Each phase has unique natural endogenous hormone levels and these hormonal fluctuations may easily be detected in peripheral blood by using routine tests. Whether these fluctuations have effects on chromosome sensitivity is the main subject of this study. Despite the absence of an exact consensus on the direct effect of hormones on the chromosomes, all classes of hormones including the steroid group are known to affect gene transcription, either as a direct transcription factor or through activation transcription factor (Nussey and Whitehead, 2001). While androgens increase apoptosis, estrogens have been determined to have a protective effect on cell death (Cutolo et al., 2005). In other words, hormones show epigenetic effects on the phenotype of the individuals through changing gene transcription levels. It is not clearly known whether alterations in gene transcription have an effect on chromosome sensitivity or not. Before analyzing the results of the studies on this topic, the effects of exogenous risk factors like environmental pollutants or exposure through diet on sex hormone levels, the main subject of the study, must also be questioned. It is known that gradually increasing the presence of environmental pollutants including cigarette smoke and pesticides in living areas leads to more exposure to them of all living things, including humans. For example, some pesticides produce clastogenic and aneugenic types of abnormalities, and as a result, reduce the mitotic index (MI) in Allium cepa root tip cells (Yüzbaşiogrulu et al., 2009). Smoking is one of the main factors causing changes in reproductive hormone levels in women (Thomford and Mattison, 1986; Barrett-Connor and Khaw, 1987; Daniel et al., 1992; Sofuoglu et al., 2001; Brand et al., 2011). Some pollutants show some
direct or indirect effects (through biotransformation) including estrogenic activities (Adami et al., 1995; Kojima et al., 2005; Lee et al., 2012; Li et al., 2012). Strong evidence and suspicions exist about the fact that exposure to exogenous estrogen-like steroids (xenoestrogen) increases different cancer types, mainly breast cancer (Stellman et al., 1998; Meek and Finch, 1999; Rudel et al., 2001; Charlier and Plomteux, 2002; Iwai et al., 2005; Meeks et al., 2012). Because of 2 common properties of breast cancer, estrogens were reported to increase genomic instability through directly stimulating DNA mutation or aneuploidy (Cavalieri and Rogan, 2002).

Estrogen is important for fertility and aids in regulating the menstrual cycle during the fertile period. However, lifelong estrogen exposure increases the risk for breast (Sæther et al., 2012) and endometrial cancer (Jaakkola et al., 2011) together. When genotoxic and cytotoxic effects of xenoestrogen in the cells are analyzed, increased cell proliferation rate was reported to bring the risks together. These risks direct the cell to a neoplastic phenotype as the accumulation of some genetic damage combinations. These types of genetic errors and neoplastic transformation were reported to be caused by hormones, drugs, infectious agents, chemicals, physical or mechanic trauma, and other chronic irritations (Preston-Martin et al., 1990). An increased sister chromatid exchange (SCE) frequency was observed in women who used ovulation-stimulating drugs for in vitro fertilization (Joseph-Lerner et al., 1993). Estrogens have been reported to induce tumors in organs of rodents, except for the liver (Lieber, 1997). Adding varying doses of 17β E2 to cultured human peripheral blood lymphocytes significantly increased the SCE number in the cell (Ahmad et al., 2000; Djeleic and Djelic, 2002). In addition, E2 induced various genetic disorders like some chromosomal and genetic lesions including aneuploidy, chromosomal aberration, gene amplification, and microsatellite instability under in vivo and in vitro conditions with various cell test systems (Lieber, 2000). The presence of indirect DNA damage related to estrogen-induced oxidation is already known, and estradiol and synthetic estrogen have been found to induce quantitative, structural chromosome aberrations and many types of gene mutations in vivo. Estrogens including estradiol and estrone are considered to be genotoxic carcinogens (Cavalieri et al., 2000). In addition, micronucleus (MN) formation arising from the chromosome-damaging effects of estradiol has been suggested. It has been suggested that induced genomic injury arises from suppression of checkpoints responsible for hemostatic control of the cell cycle (Fischer et al., 2001). In a similar study to ours (Landi and Barale, 1999), significant fluctuations were seen in SCE and chromosomal aberration (CA) frequencies related with the menstrual cycle. In that study, while SCE frequency reached the maximum value at the end of the menstrual cycle, it declined in ovulation, whereas CAs tended to gradually increase beginning from menstruation until the ovulation phase and gradually decreased thereafter. MN values did not significantly fluctuate in that study, and statistically significant differences in SCE, CA, and MN frequencies were not observed. In human peripheral lymphocytes obtained in certain phases of the menstrual cycle and cultured in vitro, SCE frequencies induced by mutagens were shown to be significantly changed by endogenous sex hormones. These hormones were reported to play a role in the suppression of cell cycle checkpoints activated after any genetic errors in the cells following mutagen treatment. This effect has been suggested to be an epigenetic mechanism related with hormone carcinogenesis (Cocchi et al., 2005). However, it must be taken into consideration that drug-metabolizing enzymes and polymorphisms in these enzyme receptors are responsible for different reactions to the same effects in cancer development in living organisms (Nebert et al., 1999).

Knowing potential periodical chromosomal sensitivities or epigenetic mechanisms over a lifetime is of great importance, in that we cannot exactly arrange the optimal time to encounter these risks. Nevertheless, even a partial arrangement of periods of drug use would provide significant benefits in overcoming a periodic chromosomal sensitivity process if present. We think that our work is very important due to the above reasons.

2. Materials and methods

In this study, peripheral blood samples of reproductive-age women obtained at 3 different times in the menstrual cycle period (follicular, ovulation, and luteal phases) were used as test material under in vitro conditions. To determine sensitivity, mitomycin C (MMC; 0.25 µg/mL) was used for 24- or 48-h periods. In the study, CA and SCE tests were used as short-term genotoxicity tests.

2.1. Method

Donors were a total of 8 healthy volunteer women, 4 nonsmokers (NS) and 4 smokers (S; have been smoking 11–20 cigarettes a day for 3–4 years) within the same age range (23–28 years), with normal body mass index (19–24.9 kg/m²), who have regular menstrual cycles, who do not use oral contraceptives, and who do not have to use any drugs due to any infectious or chronic diseases. Blood was drawn 3 times from each woman, on day 4–6 of the menstrual cycle (follicular phase), day 14–15 (ovulation phase), and day 23–24 (luteal phase). Obtained peripheral blood was subjected to hormone (FSH, LH, E2, and progesterone) concentration detection in a center accredited by the Joint Commission International (ÇUTF Balcalı Hospital, Central Laboratory); parallel samples
obtained into heparinized tubes were incubated in chromosome medium for use in the genotoxicity studies.

2.2. Mitomycin C
MMC was used as a positive genotoxic agent in this study; it is a strong DNA cross-linker. It achieves the cross link through alkylating the molecule. This chemical is used as an antitumoral agent today. The MMC (Kyowa, Hakko, Japan; Sigma CAS No: 50-07-7) used in our study was commercially provided.

2.3. In vitro SCE and CA assays
The techniques by Evans (1984) and Perry and Thompson (1984) were applied with minor modifications for the preparation of chromosomes. This study was also organized in agreement with the International Programme on Chemical Safety guidelines (Albertini et al., 2000). Eight voluntary healthy female donors were used in this study. The blood samples (0.2 mL) taken from donors were added to 2.5 mL of karyotyping medium (PB-MAX [GIBCO], Cat. No. 12552-013) for the in vitro CA test, and were supplemented with 10 µg/mL bromodeoxyuridine (Sigma, B5002) for the in vitro SCE test. These cultures were incubated at 37 °C for 72 h. The positive control culture tubes were treated with MMC (0.25 µg/mL) for 24 or 48 h. Colchicine (0.06 µg/mL, Sigma C9754) was added for the last 2 h of the cell culture in order to arrest mitosis. The frequency of SCE was investigated by the examination of 25 cells from each variable during the second metaphase. These results were used to determine the mean number of SCEs (SCEs/cell). Different types of structural and numerical aberrations were investigated by the examination of metaphases of 100 well-spread samples for each donor. Furthermore, 100 cells from each donor, a total of 800 cells, were scored for proliferation index (PI) in nonsmoker and smoker donors. The PI was calculated by the following formula: PI = [(1 × M1) + (2 × M2) + (3 × M3)] / total scored cells, where M1, M2, and M3 are the fractions of cells undergoing the first, second, and third mitosis, respectively, during the 72-h period of cell culture. MI was also determined by scoring a total of 3000 cells from each parameter.

2.4. Statistical significance
The normal distribution of the data was confirmed using the nonparametric Kolmogorov–Smirnov test. Multiple comparisons between the control and experimental groups were performed using one-way ANOVA (Bonferroni test) at P < 0.05. Chromatid and chromosome gaps were not evaluated as chromosome aberrations (Mace et al., 1978).

3. Results
3.1. Hormone levels of donors
Significant fluctuations were observed in the hormone values of donors during the menstrual cycle. An overall reduction was observed in the hormone values of smokers except in a few cases. This reduction was found to be significant, particularly in the ovulation phase (P < 0.05) (Table 1).

3.2. Effect of menstrual cycle on SCE frequency
When data of the SCE test were analyzed, the highest SCE concentration was detected in the follicular phase and the lowest SCE frequency was found in the luteal phase in untreated controls of NS donors. In the NS group, 48-h MMC treatment significantly increased SCE frequency in all 3 phases. Ovulation phase showed the maximum SCE formation reaction to MMC (P < 0.01) (Table 2).

When SCE frequencies found in untreated controls of smoking and nonsmoking donors were compared, smoking did not indicate obvious SCE induction. No significant difference occurred in other variables in terms of smoking (Table 2).

<table>
<thead>
<tr>
<th>Table 1. Mean hormone values of peripheral blood obtained from nonsmoking and smoking donors in different phases.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Phase</td>
</tr>
<tr>
<td>Follicular</td>
</tr>
<tr>
<td>Ovulation</td>
</tr>
<tr>
<td>Luteal</td>
</tr>
</tbody>
</table>

Comparing the nonsmokers and smokers, *: P < 0.05.
3.3. Effect of menstrual cycle on chromosomal abnormality

The most common chromosomal anomalies were found to be chromatid (B’) and chromosome deletion (B”) in terms of chromosomal abnormalities of the various phases of the menstrual cycle and chromosomal aberration per cell. Although the high frequency of these 2 aberrations suggests a clastogenic effect, no significant finding about aneugenity was encountered.

For the nonsmoking group, significant CA frequency was found in the follicular phase and the lowest CA was found in the luteal phase (Table 2). These findings are similar to those of SCE.

In the untreated controls of both the ovulation and luteal phases in this group, CA frequencies were found to be significantly higher than those of the NS group. These results indicate that smoking increased CA formation. Interestingly, individuals in the S group were observed to have been affected more than those in the NS group during the luteal phase (Table 2).

CA/cell frequency was found to be similar with CA findings. Maximum frequency for this value was detected also in the luteal phase (Table 2).

3.4. Cytotoxic effect

Pls of controls in the NS group were found to be similar in all 3 phases. Although MMC treatment caused a reduction in PI, it was not statistically significant (Table 3).

When PI values obtained from donors of the S group were analyzed in terms of untreated controls, the maximum PI value was found in the follicular phase and the minimum PI value was found in the ovulation phase. MMC treatment led to significant effects in the follicular phase only (Table 3).

When PI values in both the S and NS groups were analyzed, the minimum MI value was detected in the ovulation phase and the maximum value was found in the luteal phase. Significant differences were found between follicular phase controls (P < 0.05) (Table 3).

When MI values in controls in the S group were analyzed, the maximum value was found in the follicular phase and the minimum value was found in the luteal phase. The MI value calculated in the follicular phase was significantly higher than the MI value calculated in the ovulation and luteal phases (P < 0.05), although MMC treatment decreased MI values in this group significantly (Table 3).

Smoking habit caused significant increase in MI values in the follicular phase (Table 3).

### Table 2. SCE frequencies, abnormal cell percentage, and CA/cell ratio in peripheral blood obtained in various phases of the menstrual cycle in nonsmokers and smokers.

<table>
<thead>
<tr>
<th>Monthly periods</th>
<th>Treatment</th>
<th>SCE/cell ± SE</th>
<th>% Abnormal cell ± SE</th>
<th>CA/cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Variables</td>
<td>Time (h)</td>
<td>Nonsmokers</td>
<td>Smokers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fol. phase</td>
<td>Control</td>
<td>24</td>
<td>10.26 ± 1.07</td>
<td>12.29 ± 1.40</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>48</td>
<td>17.06 ± 2.06</td>
<td>18.52 ± 2.46</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>24</td>
<td>8.79 ± 0.23</td>
<td>8.13 ± 1.04</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>48</td>
<td>21.68 ± 3.43</td>
<td>16.59 ± 0.85</td>
</tr>
<tr>
<td>Lat. phase</td>
<td>Control</td>
<td>24</td>
<td>8.33 ± 0.83</td>
<td>7.76 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>48</td>
<td>13.06 ± 2.25</td>
<td>12.26 ± 1.05</td>
</tr>
</tbody>
</table>

a: Compared with own control group.  
b: Compared with analog in follicular phase.  
c: Compared with analog in ovulation phase.  
d: Compared with the non-smokers and smokers.
4. Discussion
Potential genotoxicity findings related to the menstrual cycle must be taken into consideration along with exposure to environmental risk factors in life. Data on which phase (follicular, ovulation, and luteal phases) carries more risk for chromosome sensitivity or which phase is more stable are of great importance for the health of the individual, and these data may be evaluated in the context of preventive health. The menstrual cycle, a physiologic event for reproductive-age women, is a process repeated every 28 days until menopause except during periods of pregnancy. Significant hormonal fluctuations occur in endogenous sex hormones (E2, FSH, LH, and progesterone). These fluctuations are known to cause some genetic and/or epigenetic effects in cells (Fowden and Forhead, 2009). Hormones have been detected to be a direct transcription factor (Nussey and Whitehead, 2001) and have been shown to have a protective effect against cell death (Cutolo et al., 2005). In addition, estrogen was reported to increase genomic imbalance through directly stimulating DNA mutation or aneuploidy, and this also increases breast cancer risk related with initiation of oxidative damage (Cavalieri and Rogan, 2002). In a similar study (Landi and Barale, 1999), significant fluctuations were observed in SCE and CA related with the menstrual cycle. In that study, while SCE reached its maximum value at the end of the menstrual cycle, it declined during ovulation, whereas CA tended to gradually increase beginning from menstruation to the ovulation phase and it decreased gradually thereafter. In the same study, MN did not significantly fluctuate. In that study, it was emphasized that changes occurred in genotoxicity findings related with endogenous sex hormone fluctuations, and significant fluctuations were also detected in our study. However, while these results are consistent with those of our study in some aspects, they are also inconsistent with some parts of our study. In our study, the severity of cellular reaction to MMC treatment was found to be as important as the untreated control findings, because these results enable us to estimate the outcomes of exposure to environmental risk factors at any stage of the menstrual cycle. We want to draw attention to hormone level increases in the ovulation phase in the NS group and in the luteal phase in the S group before analyzing the results of the tests applied as the indicator of mutagenity.

The proliferative effect of smoking that we consider to arise from suppressing checkpoints of the cell cycle was particularly observed in the follicular phase, and this effect indicates the tumorigenic and carcinogenic properties of smoking. In summary, it was put forward that the most sensitive phases in terms of SCE and CA response to MMC treatment were the follicular and ovulation phases—the first half of the menstrual cycle—and the most stable phase was the luteal phase. We consider that the results of our study should be supported with other studies and additional test systems.

Acknowledgments
This work was supported by the Çukurova University Research Fund, FEF2009YL49.

Table 3. Cytotoxic effects in peripheral blood obtained in various phases of the menstrual cycle in nonsmokers and smokers.

<table>
<thead>
<tr>
<th>Monthly periods</th>
<th>Treatment</th>
<th>PI ± SE</th>
<th>MI ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Variables</td>
<td>Time (h)</td>
<td>Nonsmokers</td>
</tr>
<tr>
<td>Fol. phase</td>
<td>Control</td>
<td>-</td>
<td>1.80 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>MMC 24</td>
<td></td>
<td>1.63 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>MMC 48</td>
<td></td>
<td>1.57 ± 0.14</td>
</tr>
<tr>
<td>Ovu. phase</td>
<td>Control</td>
<td>-</td>
<td>1.84 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>MMC 24</td>
<td></td>
<td>1.70 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>MMC 48</td>
<td></td>
<td>1.69 ± 0.11</td>
</tr>
<tr>
<td>Lut. phase</td>
<td>Control</td>
<td>-</td>
<td>1.77 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>MMC 24</td>
<td></td>
<td>1.62 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>MMC 48</td>
<td></td>
<td>1.63 ± 0.06</td>
</tr>
</tbody>
</table>

a: Compared with own control group.  
a1 b1 c1 d1 : P < 0.05  
b: Compared with analog in follicular phase.  
a2 b2 c2 d2 : P < 0.01  
c: Compared with analog in ovulation phase.  
a3 b3 c3 d3 : P < 0.001  
d: Compared with the non-smokers and smokers.
References


