Genetic disorder induced by the oral contraceptive drug “Triocept”

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Abstract

The purpose of this study was to investigate the capability of the oral contraceptive drug "Triocept" (0.05mg levonorgestrel + 0.03mg ethinylestradiol) in inducing primary genetic damages in human chromosome by analyzing the in vitro induction of sister chromatid exchanges (SCEs) and clastogenic activity by chromosomal abnormalities in human lymphocyte culture. Five different Triocept concentrations that correspond to 0.5, 1, 2, 5 and 10 folds of the daily therapeutic dose (DTD) were used. Bromodeoxyuridine substituted DNA was stained by Florecente plus Giemsa (FPG) technique. The results showed that the SCE averages were increased by increasing the Triocept concentration, and gave significant differences not only, between control and the concentrations, but also between concentrations themselves. On the other hand, the results indicated that, there were four main different types of chromosomal aberrations (stickiness, fragments, deletions and polyploidy). The total aberrant metaphase percentages (5.5, 11.5, 30.2, 42.5 and 89.6%) were increased by increasing the drug concentrations, and stickiness proven was to be the highest percentages (4.5, 6.5, 18.2, 22.5 and 45.6%). This increased of chromosomal aberrations with the high concentrations of the drug gave evidence that the tested drug is effective in interfering with spindle fibers and/or spindle formation. In conclusion, the results suggested that the contraceptive drug "Triocept" is capable to cause primary damage in genetic material according to its dose, so it is recommended that low dose oral Triocept might be used under medical supervision.

Key wards: Chromosomal aberrations, genetic disorder, human lymphocytes, oral contraceptive drug, sister chromatid exchanges (SCEs), and Triocept.

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Introduction

Oral contraceptives or birth control pills are used by women to prevent pregnancy, to regulate the menstrual cycle, and to provide hormone replacement therapy. There are two basic of birth control pills: 1) combination pills, which contain the hormones estrogen and progestin, and 2) progestin-only pills. Combined birth control pills work mainly by stopping ovulation. The pill may have adverse interactions with some other medications. The combination birth control pill has health benefits in addition to preventing pregnancy, since it may decrease the risk of uterus and ovary cancer and improve bone density during perimenopause. Long-term benefits include reduced rates of endometrial, ovarian, and colorectal cancer. The most serious risk of combined contraceptive, including the pill, is the potential for cardiovascular complications: blood clots, stroke, hypertension, or heart attack. The risk is higher in some women, including women older than 35 years who smoke more than 15 cigarettes a day or women who have multiple risk factors for cardiovascular disease, such as high cholesterol, high blood pressure, and diabetes (University Health Services, 2009 and ACOG Practice Bulletin No. 110, 2010).

Trioccept pills, as one of the contraceptive drugs, prevent eggs from maturing to the point at which they can be fertilized. In addition, the cervical mucus remains thick, which makes it difficult for man’s sperms to ascend. Trioccept thus offers multiple protections against pregnancy. The high degree of reliability is equal to that of the classical, single phase combined preparation and is far superior to any other method of contraception (Manufacturer; Chemical Industries Development, CID).

Genotoxins are agents specifically producing genetic alterations at sub-toxic exposure levels, which result in organisms with altered hereditary characteristics. Depending upon the developmental stage of an individual, a genotoxin can exert teratogenic effect or cause mutations not only in somatic cells but also in germinal cells. Mutational damage results in situation where not only an exposed person has the possibility of deleterious effects, but also his progeny, generation upon generation (Hafez, 1991).

The ideal genetic assay for occupational monitoring would be rapid, inexpensive, highly objective, and predictive (Shirazu, 1980 and Tezuka et al., 1980). The analysis of chromosomes from human leucocytes cultures has become one of the main methods in genetic toxicology and clinical cytogenetic. The culture of leucocytes is inexpensive, simple to perform, reliable, and therefore, suitable for routine work, so it has been widely used by several investigators (Kram et al., 1979; Inoue

Sister chromatid exchange (SCE) is a classic toxicology assay for genotoxicity and for detecting alterations to the biochemistry underlying cellular homologous recombination (Stults et al., 2014). SCE analysis appears to be a very good screening tool for evaluation of primary genetic damage induced by contaminants and/or pollutants. So, it is consider as one of the early studies on hospital patients (Lezana et al., 1977). Analysis of SCE in human cells may also play a role in establishing the dose received, or at least in identifying affected individuals following accidental exposure to know genotoxic substances. This analysis is rapid, sensitive and can be completed very shortly after the actual exposure has occurred (about 2-3 days) (Brusick, 1986).

This work aims at studying the possible capability of the oral drug triocept to induce genetic disorder by employing: 1) in vitro Induction of sister chromatid exchanges (SCEs); and 2) Analysis of chromosomal abnormalities in human lymphocyte culture.

**Materials and Methods**

This study was done at the Department of Molecular Biology, Genetic Engineering & Biotechnology Research Institute, Sadat City University, Egypt; and Genetics Department, Faculty of Agriculture, Alexandria University, Egypt.

**Materials:**

In this study, the oral contraceptive pills “Triocept” was used, since it produced by Chemical Industries Development (CID) Company in the form of tablets. The memo-pack contains:

- 6 tablets each with 0.05 mg levonorgestrel (LN) + 0.03 mg ethinylestradiol (ES)
- 5 tablets each with 0.075 mg LN + 0.04 mg ES
- 10 tablets each with 0.125 mg LN + 0.03 mg ES

Table (1) represents the drug concentration and its correspond to the daily therapeutic dose (DTD).
Table (1): The concentration of Triocept and its correspond to the daily therapeutic dose (DTD).

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Triocept Concentrations (µg/ml)</th>
<th>Code</th>
<th>Folds of the daily therapeutic dose (DTD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.25 LN + 0.15 Es</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>0.5 LN + 0.3 Es</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1.0 LN + 0.6 Es</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>2.5 LN + 1.5 Es</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>5.0 LN + 3.0 Es</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

LN: levonorgestrel; and Es: ethinylestradiol

Methods:

**In vitro induction of sister chromatid exchanges (SCEs) in human lymphocytes:**

Heparinized venous blood was collected from normal healthy adult women. Human karyotyping medium purchased from GIBCO (USA) was used in this assay. Analysis of SCEs was carried out according to Schwerzacher (1974) and Seehy (2007). The culture medium was divided into 2 groups; control and 5 concentrations (Table, 1) of Triocept drug. The cells in cultures were exposed to 5-bromo-2'-deoxyuridine (BrdU 100 µg/ml; Sigma) after 24 hrs of the initiation of cultures. The cultures were incubated in tightly sealed tubes at 37 °C for 72 hrs. Two hrs prior to harvesting, 0.1 ml colcemid was added for each culture and re-incubated for two hrs at 37 °C. The cultures were centrifuged for 8 min at 1200 rpm, the supernatant was discarded and the cell pellet was re-suspended with the last drop of supernatant, then 8 ml of pre-wormed (37 °C) hypotonic solution (0.075 M KCl) were added, and left for 10 min at 37 °C, then centrifuged for 5 min at 1200 rpm. The cell pellet was fixed for an hour in about 8 ml freshly prepared fixative fluid (3 parts methanol : 1 part glacial acetic acid) and centrifuged. The cell pellet was fixed three times for 10 min each.

Human chromosomes were stained for SCEs using Florence plus Giemsa (FPG) according to Goto *et al.* (1978). The cells were photographed and SCEs were counted from the microscope images of second metaphase, and then the analysis of variance using F- test was applied. To evaluate the differences in mean SCE frequencies between treated and control groups, Duncan’s multiple range test was used (Snedecor, 1958).
Analysis of chromosomal abnormalities in Human lymphocytes:

In order to study the activity of the contraceptive drug (Triocept) to induce chromosomal abnormalities in human karyotype, the same procedure of SCEs was used with some modifications; the drug was added to culture medium at zero time of incubation, BrdU was omitted, and Human chromosomes were stained using 10% Gemisa. The chromosomes were screened for the presence of breaks, gaps, deletions and fragments ……etc.

Results

In vitro induction of Sister Chromatid Exchanges in human lymphocytes:

Table (2) shows the averages of SCEs obtained after cytological examination of human lymphocytes treated with the different concentrations of the oral contraceptive “Triocept“. Total number of 100 cells was counted with the control as well as with all tested concentrations. The least SCEs average (2.2±0.2) was detected with the control, whereas it increased after treatment to be ranged from 4.46±0.3 to 12.82±1.2. Analysis of variance using F-test showed that the tested concentrations (1, 2, 3, 4 and 5) were proven to be positive in inducing significant increases in SCEs (4.46±0.3, 5.82±0.24, 8.28±0.42, 8.64±0.63 and 12.82±1.2), respectively (Figure, 1).

Duncan’s multiple range test for analysis of mean differences was conducted as shown in table (3). It indicated that, there were significant differences between control and Triocept treatment concentrations, whereas it showed the highest mean difference was found (10.62) between the control and the highest concentration (5) of Triocept. In addition, there were significant differences between the different Triocept concentrations, since the highest difference (8.36) was between Triocept concentrations (5 and 1).

Table (2): Averages of sister chromatid exchanges in human chromosomes after treatment with “Tiocept”.

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Total cells counted</th>
<th>X ± S.E.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>2.2 ± 0.2</td>
<td>0 - 4</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>*4.46 ± 0.3</td>
<td>2 - 6</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>5.82 ± 0.24</td>
<td>3 - 8</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>*8.28 ± 0.42</td>
<td>3 - 10</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>8.64 ± 0.63</td>
<td>5 - 12</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>*12.82 ± 1.2</td>
<td>6 - 18</td>
</tr>
</tbody>
</table>

* Significant at 0.05 level probability
Table (3): Duncan’s multiple range test for mean differences of SCEs.

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>$\bar{X}$</th>
<th>$\bar{X} - \bar{X}C$</th>
<th>$\bar{X} - \bar{X}1$</th>
<th>$\bar{X} - \bar{X}2$</th>
<th>$\bar{X} - \bar{X}3$</th>
<th>$\bar{X} - \bar{X}4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>12.82</td>
<td>*10.62</td>
<td>*8.36</td>
<td>*7.00</td>
<td>*4.54</td>
<td>*4.18</td>
</tr>
<tr>
<td>4</td>
<td>8.64</td>
<td>*6.44</td>
<td>*4.18</td>
<td>*2.82</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8.28</td>
<td>6.08</td>
<td>3.82</td>
<td>*2.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.82</td>
<td>*3.62</td>
<td>1.36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.46</td>
<td>2.26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant at 0.05 level of probability

Figure (1): Photomicrograph showing human metaphase stage with in vitro induction of sister chromatid exchange (a: control, and triocept concentrations; b: 0.25 µg/ml LN+0.15 µg/ml Es, c: 0.5 µg/ml LN+0.3 µg/ml Es, d: 1.0 µg/ml LN+0.6 µg/ml Es, e: 2.5 µg/ml LN+1.5 µg/ml Es and f: 5.0 µg/ml LN+3.0 µg/ml Es)
Analysis of chromosomal abnormalities in human lymphocytes:

The analysis of chromosomal abnormalities in human lymphocytes after treatment with different concentrations of the oral contraceptive drug "Triocept", is given in table (4) and figures (2 & 3). In this experiment, 200 cells were counted for each treatment as well as for the control group. Results showed that, there were four main types of chromosomal aberrations (stickiness, fragments, deletions and polyploidy). The total percentage of aberrant metaphase in the control was 2%, while it was 5.5, 11.5, 30.2, 42.5 and 89.6% with Triocept concentrations 1, 2, 3, 4 and 5, respectively.

For each aberrant metaphases percentages, the results revealed that, stickiness (4.5, 6.5, 18.2, 22.5 and 45.6%), fragments (0, 1, 4, 8 and 21%), deletions (1, 4, 6, 8 and 17%) and polyploidy (0, 0, 2, 4 and 6%) were increased by increasing the Triocept concentration (1, 2, 3, 4 and 5), respectively. However, stickiness represented the highest metaphase aberrant percentages.

Table (4): Chromosomal aberrations induced in human chromosomes after treatment with "Triocept".

<table>
<thead>
<tr>
<th>Concentrations Code</th>
<th>Total cell</th>
<th>Stickiness</th>
<th>Fragment</th>
<th>Deletion</th>
<th>Polyploidy</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>200</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>4.5</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>5.5</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>6.5</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>11.5</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>18.2</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>30.2</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>22.5</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>42.5</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>45.6</td>
<td>21</td>
<td>17</td>
<td>6</td>
<td>89.6</td>
</tr>
</tbody>
</table>
Figure (2): Photomicrograph showing human metaphase stage with (a: Normal, b: control and trioscept concentrations; c: 0.25 µg/ml LN+0.15 µg/ml Es, d: 0.5 µg/ml LN+0.3 µg/ml Es, and e: 1.0 µg/ml LN+0.6 µg/ml Es) where; CdG: Chromatid Gap, CEx: Chromosome Exchange, EEA: End to End Association, F: Fragment and S: Stickiness,
Figure (3): Photomicrograph showing human metaphase stage with triocept concentrations (g: 0.5 µg/ml LN + 0.3 µg/ml Es, h: 0.5 µg/ml LN + 0.3 µg/ml Es, i: 1.0 µg/ml LN + 0.6 µg/ml Es and j: 2.5 µg/ml LN + 1.5 µg/ml Es; k: 2.5 µg/ml LN + 1.5 µg/ml Es and l: 5.0 µg/ml LN + 3.0 µg/ml Es) where; CdD: Chromatid Deletion, CdG: Chromatid Gap, CEx: Chromosome Exchange, CmD: Chromosome Deletion, EEA: End to End Association, F: Fragment, poly: polyploidy, and S: Stickiness.

Discussion

In this study, 5 different concentrations (0.25 µg/ml LN+0.15 µg/ml Es, 0.5 µg/ml LN+0.3 µg/ml Es, 1.0 µg/ml LN+0.6 µg/ml Es, 2.5 µg/ml LN+1.5 µg/ml Es and 5.0 µg/ml LN+3.0 µg/ml Es) of Triocept were used and tested. They correspond to 0.5, 1, 2, 5 and 10 folds of DTD, respectively. The results indicated that the use of contraceptive drug "Triocept" is associated with induction of chromosomal abnormalities. The analysis of variance using F-test for SCE frequencies after treatment with Triocept concentrations showed that, there was a significant differences and the highest SCE average (12.82) was with the highest concentration (5) of Triocept. The average was increased (4.46,
5.82, 8.28, 8.64 and 12.82) by increasing the Triocept concentrations. Duncan's multiple range test showed significant differences between the control group and the Triocept concentrations. In addition, there were significant differences between the concentrations themselves giving a concentration response relationship.

On the other hand, the results showed that the total percentages of aberrant metaphase (6.5, 10.5, 30.2, 42.5 and 89.6%) were increased by increasing the Triocept concentrations (1, 2, 3, 4 and 5), respectively. Stickiness gave the highest percentages of aberrant metaphase (4.5, 6.5, 18.2, 22.5 and 45.6%) with the five Triocept concentrations, respectively. However, the obtained results gave evidence that the tested concentrations were positive in causing primary DNA damage and clastogenic effect as well.

These results are in agreement with those reported by other authors (Kabarity and Khodari, 1967; Badr and Badr, 1969; Stenchever et al., 1969; Timson, 1969; McQuarrie et al., 1970; and Badr et al., 1973) as they indicated that contraceptive drugs of steroid nature induce structural chromosomal aberration both in vivo and in vitro, and that these differ according to the type and aspect of organism studied. Oral contraceptive pills are associated with a number of side effects ranging from the relatively minor to increased risk of death from several causers (Gilman et al., 1985). Recently, it was found that prolonged use of estrogen containing contraceptive medication at an early stage may be associated with risk of breast cancer (Liehr, 1990).

Concerning the effect of oral contraceptive drugs upon chromosomes, data from several reports have suggested that exogenous estrogens and progestogens may cause cytogenetic aberrations in lymphocytes from women taking oral contraceptive drugs (Goh, 1967; Badr et al., 1973 and Seehy & Hafez, 1992), whereas other studies have not demonstrated increased chromosome breakages in pill users (Shapiro et al., 1972 and de Gutierrez & Lisker, 1973). In addition, experiments on rats, mice, dogs, hamsters and human indicated that these compounds may induce cytogenetic abnormalities in germ cells (Littlefield et al., 1975; and Seehy & Hafez, 1992).

Induction of SCEs has been demonstrated to be a sensitive indicator of DNA damage induced by chemical mutagens in eukaryotic cells cultured in vitro (Kato, 1974 and Seehy, 2007) and it is proposed that analysis of SCE induction in vivo may provide a useful technique for the screening of mutagenic and carcinogenic compounds.

In conclusion, the present study indicated that the contraceptive drug "Triocept" is a positive inducer of primary genetic damage. In addition, clastogenic effect as detected for this drug.
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References


Chemical Industries Development (CID). Triocpt. Bach No. 05150003


