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# Evaluation of the genotoxicity of clomiphene citrate

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ABSTRACT

Clomiphene citrate (CC) is a selective estrogen-receptor modulator that is primarily used to enhance follicular development in women receiving *in vitro* fertilization (IVF) treatment. Although some studies suggested large increases in ovarian cancer risk related to fertility medications, this association has not been confirmed in other studies. Whether there could be a residual, small risk is still an open question. It is known that genomic instability and multiple genetic changes may be required in carcinogenesis. Genomic instability such as single-base changes, chromosomal rearrangements or aneuploidy may accelerate this process. Genomic instability is not only central to carcinogenesis, but it is also a factor in some neurodegenerative diseases such as amyotrophic lateral sclerosis or the neuromuscular disease myotonic dystrophy. For these reasons, this study was planned to examine genotoxic effects of CC in human lymphocytes by use of the chromosome aberration (CA) assay, the micronucleus (MN) test, the comet assay, and the test for bacterial mutagenicity in *Salmonella typhimurium* strains TA98 and TA100 (Ames test). Concentrations of 0.40, 0.80, 1.60, and 3.20 μg/ml of CC significantly increased the frequency of chromosomal aberrations ($p < 0.01$ and $p < 0.001$) and micronuclei ($p < 0.05$, $p < 0.01$ and $p < 0.001$) in cultured human lymphocytes, and of DNA damage (tail length, $p < 0.05$, except 0.80 μg/ml) in isolated lymphocytes compared with their respective controls. The highest CC concentration at 24 h and highest two concentrations after the 48-h treatment significantly decreased the mitotic index. The Ames test showed that the concentrations of CC used in this study induced neither base-pair substitutions nor frame-shift mutations in *S. typhimurium* strains TA98 and TA100.



## 1. Introduction

Ovulation-inducing drugs, especially clomiphene citrate (CC) and gonadotropins, have been widely used for infertility treatments since 1962 and their use is increasing every day [1]. CC is the first choice for treatment of women with ovulatory disorders who are normally estrogenized, i.e. predominantly those with polycystic ovaries (PCO) [1]. CC is a selective estrogen-receptor modulator that is primarily used to enhance follicular development and induce ovulation in women undergoing *in vitro* fertilization (IVF) [2–4]. Chemically, CC is a non-steroidal triphenylethylene derivative that exhibits both estrogen agonist and antagonist properties [5]. It increases serum estradiol levels during the follicular phase of the menstrual cycle [6]. CC is administered orally, typically starting on the third to fifth day after the onset of spontaneous or progestin-induced menses. Treatment begins with a single 50-mg tablet daily for 5 consecutive days. The effective dose of CC ranges from 50 to 250 mg/day; doses in excess of 100 mg/day are not approved by the FDA [7]. Lower doses (12.5 and 25 mg/day) are used in trials with women who demonstrate exquisite sensitivity to CC or consistently develop large ovarian cysts that interfere with efficient cyclic treatment [7,8].

Although there are conflicting results about the relations between fertility treatments and some types of cancer [9–15], some authors have reported that fertility treatments (especially gonadotropins and CC) are associated with various malignancies including endometrial, ovarian and breast cancer [16–20]. Genomic instability, such as single-base changes, chromosomal rearrangements or aneuploidy, is a characteristic of many cancers and may accelerate the cancer process. Among the experimental methods used for evaluation of the genotoxic potential of chemicals, the most important are the chromosome aberration test, the micronucleus test and the comet assay in human lymphocytes, and the reverse mutation assay in bacteria. They are used as sensitive and relatively simple assays in evaluating genotoxicity and potential carcinogenicity of different chemicals to which humans are exposed [21–27].

To the best of our knowledge, only a few studies so far have been carried out on the effect of CC in eukaryotic and bacterial systems. Ohnishi et al. [28] reported that clomid which contains CC,

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induced DNA-strand breaks and SOS responses such as mutation and expression of the *umuC*$^{+}$ gene in *Escherichia coli* [28]. Arriaga-Alba et al. [29] stated that CC induced frame-shift mutations in the *Salmonella typhimurium* TA1538, TA97 and TA100 strains *in vitro* in the presence of metabolic activation (S9). However, CC did not induce base-pair substitution mutations. CC induced genolethal DNA damage in *E. coli* PolA$^{-}$/PolA$^{+}$ in the pre-incubation method with S9, and in the disk-diffusion method without S9 [29]. London et al. [4] reported that CC reduced the number of ovulated oocytes and significantly ($p < 0.05$) increased the hyperploidy at 100 mg/kg bw *in vivo* in mice. Similarly, a 5.0-μg/ml concentration of CC significantly ($p < 0.05$) increased the level of hyperploidy and reduced the proportion of metaphase-I oocytes in the mouse [4]. Duran et al. [30] investigated the potential genotoxicity of CC in rat reticulocytes with the micronucleus assay. At concentrations of 0.16, 0.32 and 0.64 mg/kg bw, CC was administrated to rats intraperitoneally, daily for 5 days. This treatment caused a dose-dependent increase in genomic instability and micronucleus frequency in bone-marrow stem cells [30].

The purpose of this study was to evaluate genotoxic potential of the ovulation-stimulating drug CC by use of the chromosome aberration test, the micronucleus test, and the comet assay in human lymphocytes, as well as the Ames assay in *S. typhimurium* strains TA98 and TA100. In view of controversial results about the carcinogenic potential of CC, this study may reveal genomic instability such as changes in DNA bases, chromosomal rearrangements or aneuploidy, which may accelerate carcinogenesis.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Test substances clomiphene citrate (CAS No: 298-02-2) and L-histidine (CAS No: 71-00-1) were obtained from Fluka. Chromosome medium B was obtained from Biochrome. Mitomycin C (CAS No: 200-008-6), cytochalasin B (CAS No: 14930-96-2), NaCl (CAS No: 7647-14-5), biotine (CAS No: 58-85-5), ampicillin trihydrate (CAS No: 7177-48-2), 2-aminofluorene (CAS No: 153-78-6), $Na_2HPO_4{\cdot}7H_2O$ (CAS No: 7782-85-6), 4-nitro-*o*-phenylenediamine (CAS No: 99-56-9), S9 microsomal fraction (CAS No: S2442), Glucose-6-phosphate (CAS No: 9001-40-5) were obtained from Sigma. EDTA (CAS No: 6381-92-6), NaOH (CAS No: 1310-73-2), Tris (CAS No: 77-86-1), Triton X-100 (CAS No: 9002-93-1), DMSO (CAS No: 67-68-5), low-melting agarose (CAS No: 9012-36-6), normal-melting agarose (CAS No: 9012-36-6), EtBr (CAS No: 1239-48-8) were obtained from Applichem. Citric acid monohydrate (CAS No: 100243), NaCl (CAS No: 106400), KCl (CAS No: 529552), sodium azide (CAS No: 822335), $K_2HPO_4$ (CAS No: 105109), $NaH_2PO_4{\cdot}2H_2O$ (CAS No: 137018), $MgCl_2{\cdot}6H_2O$ (CAS No: 442611), $MgSO_4{\cdot}7H_2O$ (CAS No: 105886) were obtained from Merck; Nutrient broth (CAS No: LAB014), agar (CAS No: Q31114/218) were obtained from Lab M; $Na(NH_4)HPO_4{\cdot}4H_2O$ (CAS No: 60200) was obtained from Riedel.

### 2.2. Chromosome aberration (CA) test

Peripheral blood samples were collected from four healthy non-smoking female donors (aged 24–26 years), who were free of any known exposure to genotoxic agents. Whole blood was cultured in chromosome medium B and incubated at 37 °C for 72 h. Duplicate cultures were used at each concentration. Test substances were added after 24 h and 48 h of culture initiation, and colchicine (0.06 μg/ml) was added to each culture at 2 h before harvesting. Human lymphocytes were exposed to four concentrations (0.40, 0.80, 1.60, 3.20 μg/ml) of CC, which were equivalent to CC doses of 25, 50, 100, and 200 mg/day for average 60-kg females. A negative (distilled water) and a positive control (mitomycin-C, MMC, 0.20 μg/ml) were incubated simultaneously with the cultures with test substances.

At the end of the incubation the cells were collected by centrifugation at 1200 rpm for 10 min, re-suspended in a hypotonic solution (KCl, 0.075 M) for 30 min at 37 °C and then fixed in cold methanol–acetic acid (3:1) for 20 min at room temperature. This fixation step was repeated 3 times. Chromosome spreads were prepared by dropping the concentrated cell suspension onto slides.

For chromosome aberrations, air-dried slides were stained with 5% Giemsa (pH. 6.8) for 20 min and mounted with depex. Chromosome aberrations were evaluated in 100-well-spread metaphases per donor (totally 400 metaphases per concentration). The mean frequency of abnormal cells (structural and numerical chromosomal aberrations) and the number of aberrations per cell (CA/cell) were calculated.

### 2.3. Micronucleus assay

Human lymphocyte cultures were incubated at 37 °C for 72 h and cytochalasin B (Cyt-B) was added after 44 h of incubation at the final concentration of 5.2 μg/ml to arrest cytokinesis. The CC concentrations (0.40, 0.80, 1.60, 3.20 μg/ml) were added 24 and 48 h after PHA stimulation. Cultures were harvested at 72 h. The cells were treated with hypotonic solution (0.075 M KCI for 5 min) and fixed with methanol: glacial acetic acid (3:1, v/v) supplemented with formaldehyde. The slides were stained with 5% Giemsa. Micronuclei were scored from 1000 binucleated cells per donor (totally 4000 binucleated cells per concentration).

### 2.4. Comet assay

#### 2.4.1. Leukocyte isolation and in-vitro treatment

Peripheral blood samples were obtained from four healthy non-smoking female donors (aged 24–26 years) without indication of exposure to any chemical. Peripheral blood was obtained with a heparinized syringe immediately before the performance of the test. PBL were isolated with Biocoll separating solution. Cell viability was measured by means of the Trypan-blue exclusion test; viable cells have clear cytoplasm, whereas non-viable cells have a blue cytoplasm [31]. In this study, cell viability was >98%. Isolated lymphocytes were exposed to CC at 0.40, 0.80, 1.60, 3.20 μg/ml, at 37 °C for 1 h. A negative (distilled water) and a positive control ($H_2O_2$ 100 μM) were also used. After incubation, lymphocytes were centrifuged at 3000 rpm for 5 min, and then supernatant was removed and re-suspended in PBS.

#### 2.4.2. Alkaline single-cell gel-electrophoresis

The comet assay was performed under alkaline conditions according to the original procedure [32] with some modifications [22,25]. Treated cells were suspended in low melting-point agarose (0.65%) and 75 μl of this suspension was quickly layered onto slides previously pre-coated with normal-melting agarose (0.65%). Slides were immediately covered with a cover slip and placed on ice for 10–15 min. After removal of the cover slip, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, to which 10% DMSO and 1% Triton X-100 were added) at 4 °C for 1 h. Then the slides were placed on a horizontal gel-electrophoresis platform and covered with electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). The slides were left for 20 min to allow the unwinding of the DNA and then they were electrophoresed at 25 V, 300 mA for 20 min in the dark. The slides were rinsed with neutralization buffer (0.4 M Tris, pH 7.5) and stained with 50 μl of ethidium bromide.

*2.4.2.1. Image analysis and comet scoring.* The slides were examined with a fluorescence microscope (Olympus) equipped with an excitation filter of 546 nm and a barrier filter of 590 nm, at 400× magnification. Two slides were prepared for each concentration of CC. The tail length and tail intensity (%) of one hundred comets on each slide were determined (totally 400 comets per concentration, duplicate slides per treatment) with a specialized image-analysis system ("Comet Assay IV", Perceptive Instruments Ltd., UK).

### 2.5. Mitotic index and nuclear division index

The mitotic index (MI) and cytokinesis-block proliferation index were determined in cultured lymphocytes. The mitotic index (MI, number of metaphases/total interphases + metaphases) was scored by recording the number of metaphases in 1000 cells from each donor. MI was calculated according to the OECD guideline [33] and Holland et al. [34]. Five hundred (total 2000) lymphocytes were scored to evaluate the percentage of cells (CBPI) with 1, 2, 3 and 4 nuclei. The nuclear division index (NDI) was calculated according to Surralés et al. [31] as follows: $[1 \times N_1] + [2 \times N_2] + [3 \times (N_3 + N_4)]/N$ where $N_1$–$N_4$ represent the number of cells with 1–4 nuclei, respectively, and $N$ is the total number of cells analyzed.

### 2.6. Bacterial reverse-mutation assay (Ames test)

The Ames test was carried out as standard plate-incorporation test [27] with *S. typhimurium* strains TA98 and TA100, with and without S9. Four concentrations of CC (1.40, 2.80, 5.60 and 11.20 μg/plate) were used in two parallel independent experiments. CC was dissolved in sterile double-distilled water. The results were evaluated as positive if the test sample produced reverse mutations at a frequency of at least two-fold above the negative control. Sodium azide was used as a positive control for TA100 and 4-nitro-*o*-phenylenediamine for TA98 in the experiments without S9 mix, while 2-aminoflourene was used as a positive control in the experiments with S9 mix, for both strains.

### 2.7. Statistical analysis

*Z*-test was used to assess statistical significance of differences in the percentage of abnormal cells, the number of CA/cell, the MI, and the frequency of MN. Dose–response relationship was determined from the correlation and regression coefficient for the percentage of abnormal cell, CA/cell and mean MN. For each concentration 400 comets (100 comets per slide, duplicate slides per treatment) were