

Genotoxic potential evaluation of a cosmetic insoluble substance by the micronuclei assay

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Accepted for publication November 3, 2010.

Synopsis

An optical brightener (OB) powder (INCI: sodium silicoaluminate (and) glycidoxypopyl trimethyloxysilane/PEI-250 cross fluorescent brightener 230 salt (and) polyvinylalcohol crosspolymer) that is used in cosmetic facial products was tested for its genotoxic potential using the micronuclei test (MNT). It is a solid dry powder with an average size of 5 microns that is insoluble but dispersible in water. This study describes the exposure of cell culture to positive controls with and without enzymatic activation and to the test compound in different concentrations. We evaluated three end points: microscopic observation and quantification of micronuclei formation, and cell viability and proliferation. Both positive controls induced significant changes that were observed under the microscope and quantified. Based on its chemical nature, it was not anticipated that the test substance will degrade under the conditions of the experiments. However, the test is required to make sure that when solubilized, impurities that may be present, even at trace levels, will not induce a genotoxic effect. The test compound did not promote micronuclei formation or change the viability or proliferation rate of cells. During this study we faced challenges such as solubilization and correlating viability data to genotoxicity data. These are described in the body of the paper. We believe that with the emergence of the 7th European amendment that bans animal testing, sharing these data and the study protocol serves as a key in building the understanding of the utilization of *in vitro* studies in the safety assessment of cosmetic ingredients

INTRODUCTION

With the emergence of the 7th amendment to the EU Directive, the cosmetic and personal care industry is banned from experimenting on animals. It is therefore utilizing, studying, and validating a variety of *in vitro* and *ex vivo* protocols. One of the more important end points in the evaluation of safety is the potential of a compound to become genotoxic.

While this industry is known to produce relatively safe products, with the 7th amendment the perspective of safety has been broadened from acute, clearly perceivable, adverse effects such as irritation to the inclusion of the evaluation of long-term accumulating effects that can lead to a variety of chronic and deadly diseases such as inflammation and cancer. Moreover, the focus on *in vitro* methodologies is expanding our knowledge in understanding

biochemical pathways rather than focusing on clinical evaluation. Consequently, the cosmetic product manufacturer is focusing on *in vitro* studies to ensure safety and on studying their effects. Compounds that can change the genetic information of a living cell may lead to mutations. These can result in different type of toxic effects, ranging from cell death to the development of malignant tumors. While a variety of assays are currently in use in the area of regulatory genotoxicity, substantial *in vivo* testing is still required for the confirmation of genotoxic predictions. A mutagenic (from Latin word for *change*) compound can change the genetic information of the cell and thus increase the frequency of mutations. The term “genotoxicity” is used broadly and refers to potentially harmful effects on genetic material that are not necessarily associated with mutagenicity.

One test method recommended for the estimation of genotoxicity is known as the micronuclei assay. In the course of this study, mammalian cells are exposed to the test substance with and without metabolic activation. At predetermined time intervals after exposure, they are treated with a metaphase-arresting substance, harvested, stained, and observed under the microscope. Information is then collected both by observing the morphological changes and by determining them quantitatively as compared to the control.

This paper reports the results of a study evaluating the genotoxicity potential of a powder, optical brightener (OB), used in cosmetic products. It describes the challenges of working with a powder in a cell culture, the study rationale, the subjective morphological changes, and the quantitative evaluation of the data generated. In addition, cell metabolism as an end point of viability, proliferation, and the relationships between viability and the genetic mutations of cells is discussed.

MICRONUCLEI TEST

The chromosome is a single coiled piece of DNA with associated proteins containing many genes, regulatory elements, and other nucleotide sequences. A chromatid is one among two identical copies of DNA making up the replicated chromosomes. Two identical sister chromatids are joined by a region called the centromere. The term micronucleus describes the small nucleus that forms whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division. Testing for micronucleus formation is used to screen for potential genotoxic compounds. There are two versions of this study: *in vivo* and *in vitro*. The *in vivo* test is using mouse bone marrow or mouse peripheral blood (1). The *in vitro* method was developed as a replacement for the *in vivo* method and was evaluated to be a reliable genotoxicity test, although more compounds should be tested to build an additional database (2). In that sense, we are hoping that data shared in this paper will enrich the existing database.

This study was conducted as a modification of the OECD (Organization for Economic Cooperation & Development) Guideline number 487 protocol that was adopted in December of 2006 as a draft proposal: “*In Vitro* Micronucleus Test” (3). This test protocol was assessed by The European Centre for the Validation of Alternative Methods (ECVAM) that stated the following: “On the basis of a peer review of a weight of evidence retrospective validation, the Committee endorses the conclusion that the MNT is a scientifically valid alternative to the *in vitro* chromosome aberration assay for genotoxicity testing” (4). According to the protocol, the objective is to identify compounds that cause structural chromosome aberrations in cultured mammalian cells. These aberrations may be of two

types, chromosome and chromatid. Since the majority of genotoxic compounds are mutagens, the aberrations are normally of the chromatid type, but the chromosome type may also occur. Mutations in chromosomes and events that are mutation related are the cause of numerous human genetic disorders such as cystic fibrosis, hemophilia, and sickle cell anemia (5). In addition, there is evidence that events causing modifications in oncogenes and tumor suppressor genes are involved in the induction of cancer. While this protocol requires cytochalasin B to block cytokinesis, the method utilized in this study does not require it because it uses CHO-K1 cells lines and the OECD Guideline number 487 protocol states that use of cytochalasin B is not necessary for cell lines if proof of cell proliferation is provided.

The principle of this method is the exposure of a mammalian cell culture to a test substance with and without metabolic activation (6). Metabolic activation is required to assess potential genotoxic effects in a case where enzymatic transformation occurring *in vivo* leads to a toxic metabolite. At predetermined time intervals, the cells are treated with a test substance, harvested, and stained, and metaphase cells are analyzed microscopically for micronuclei formation.

MATERIALS AND METHODS

MATERIALS

The chemicals for the control group were purchased from Sigma Chemical Co. (St. Louis, MO). These include cyclophosphamide (CAS no. 50-18-0), ethyl methanesulfonate (CAS no. 62-50-0), and dimethyl sulfoxide (DMSO). The test substance, OB, was a product from Lipo Chemicals Inc. (Paterson, NJ). Nicotinamide adenine dinucleotide phosphate (NADP) was purchased from Fisher Scientific Co. (Suwannee, GA). Sodium phosphate buffer was obtained from Moltox (Boone, NC). Glucose 6 phosphate and Tween 20 were received from Sigma Aldrich (St Louis, MO), and 4',6- diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Sigma.

PREPARATION OF CHEMICALS

Cyclophosphamide (density: 1.479 g/cm³), and ethyl methanesulfonate (EMS) were used as positive controls to analyze the formation of micronuclei. One gram of cyclophosphamide was dissolved in 100 ml of water to give a concentration of 10 mg/ml. Five microliters of this solution was added to a volumetric flask and water was added to give the required final concentration of 10 µg/ml in the flask (2). An amount of 0.1 ml of EMS (density 1.15 g/cm³) was diluted to 1.0 ml with dimethyl sulfoxide (DMSO). A measured 17.5 µl of this solution was added to a 5-ml flask and DMSO was added to give a final concentration of 400 µg/ml. (2).

The OB powder was dispersed at 10 mg/ml in DMSO. An amount of 50 mg of this suspension was added to 5 ml of DMSO, subjected to ultrasonification for 30 minutes, and centrifuged at 2000 rpm for 15 minutes. Volumes of 100 µl and 150 µl of the supernatant were added to flasks of 25 cm² base surface area containing 5 ml of media, to give final concentrations of 0.2 mg/ml and 0.3 mg/ml, respectively. These doses were selected

in preliminary experiments based on the concentrations of DMSO that are nontoxic for the types of cells used.

To prepare the stock solution for cell staining according to the protocol for this assay (7), 5 mg of DAPI was dissolved in 1 ml of dimethylformamide and allowed to stand until the dissolution was complete. For the working solution, 4 μ l of the stock solution was added to 50 ml of phosphate buffer solution (PBS) and stored at 4°C, protected from light before the time of staining.

METABOLIC ACTIVATION SYSTEM

Inclusion of a metabolic activation system in the genotoxicity assay enables the detection of mutagenic activity for carcinogens and/or mutagens that require such transformation (i.e., cyclophosphamide). The metabolic activation system was prepared according to the method described in references (7) and (8). Aroclor-1254-induced rat liver S9 fraction was purchased from Moltax. The following chemicals were added in the order listed to get a total volume of 3 ml of S9 mix: sterile double-distilled H₂O (840 μ l); sodium phosphate buffer (0.1 M), pH 7.4 (1.5 ml); 4 mM NADP (150 μ l); 120 mM glucose-6-phosphate (22 μ l); potassium magnesium salt solution, 8 mM–33 mM (60 μ l); and rat liver fraction (3.00 μ l) to give a final concentration of 10% (v/v). The final concentration of the metabolic activator used for each test flask was 1% (v/v). In contrast to cyclophosphamide, EMS does not require metabolic activation and therefore the metabolic activation system was not used in the experiments involving EMS.

CELL LINE

Chinese hamster ovary (CHO-K1) cells were used as recommended in the OECD protocol (3) and were bought from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in ATCC-formulated F-12K medium supplemented with 10% fetal bovine serum (Fisher Chemicals, Fairlawn, NJ) and penicillin-streptomycin (100 UI/ml–100 μ g/ml). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ (v/v) in air. All experiments were performed on cells in the exponential growth phase.

EXPERIMENTAL SERIES AND CONDITIONS

About 300,000 cells were cultured with the media in each 25-cm² flask and held 24 hours before treatment. They were then incubated with the substances as indicated in Figure 1. For the series with metabolic activation, the cells were treated for three hours after which the media were replaced with fresh media and the cells were incubated for 24 hours. For the groups without S9 activation, the cells were incubated for 24 hours. All the cells were harvested at the end of 24 hours and stained to detect the presence of micronuclei. The following series of experiments was carried out: (1) media only (negative control); (2) DMSO (100 μ l) (negative control); (3) cyclophosphamide + S9 mix (10 μ g/ml) (positive control); (4) ethyl methanesulfonate (400 μ g/ml) (positive control); (5) OB (0.2 mg/ml); (6) OB (0.3 mg/ml); (7) OB (0.2 mg/ml) + S9 mix; and (8) OB (0.3 mg/ml) + S9 mix.

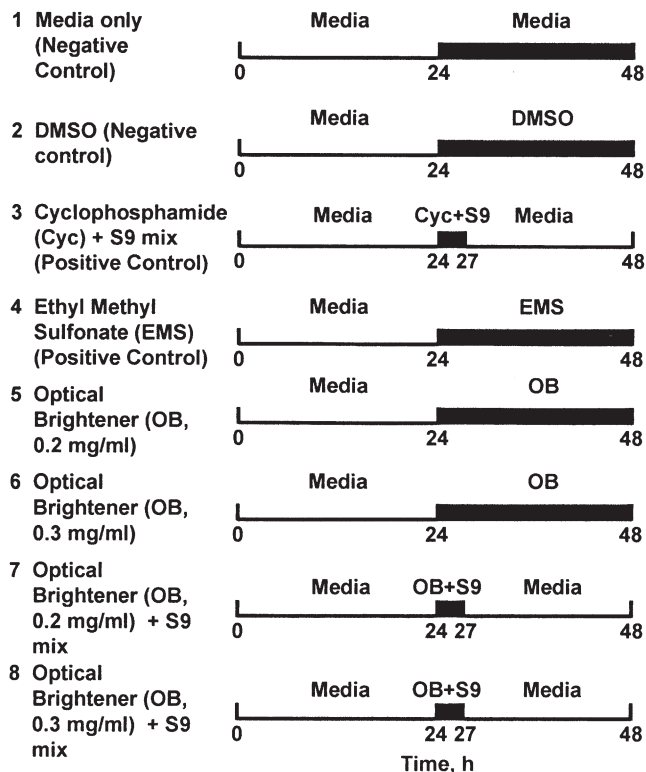


Figure 1. Scheme of experimental series and conditions. The shaded areas represent the treatment periods with tested substances or media. At the beginning of the experiments, the cells were incubated with media within 24 h. For the series with metabolic activation, the cells were treated for three hours after which the media were replaced with fresh media and the cells were incubated for 24 hours. For the groups without S9 activation, the cells were incubated for 24 hours.

All the treatment groups were set up as duplicates; the determination of cellular toxicity was made in eight independent measurements. After the end of all treatments, the cells were harvested for staining.

Cell staining. After 24 hours of incubation with the aforementioned substances, the media from all the flasks were removed. The cells were fixed by slowly adding a cold solution of 100% methanol and allowing the mixture to stand for five minutes. The methanol was removed and the cells were washed with phosphate buffer solution two times for two minutes. The cells' nuclei were then stained with 600 nM DAPI (2 ml) (4,6 diamidino-2-phenylindole) for eight minutes. This solution was removed and all the flasks were washed with PBS containing 0.05 % Tween 20 (Sigma Aldrich). The cells were kept moist by adding PBS at the end. The cells were then observed under a microscope.

Counting of micronuclei. For each experimental series, the formation of micronuclei was determined as described (9) by counting the number of micronuclei per 1000 cells using light and a fluorescent microscope (Olympus IX71, New York).

Cell viability and proliferation. A modified MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess the cytotoxicity of the studied substances as previously described (10). To measure cytotoxicity, cells were separately incubated in a

microtiter plate with each studied substance. Control cells received an equivalent volume of fresh medium containing DMSO in the same concentrations used to dissolve the OB. The duration of incubation was 24 h. On the basis of these measurements, cellular viability was calculated for each substance. A decrease in the cellular viability indicated an increase in toxicity. Cell proliferation was conducted by using a hemacytometer (Veeder-Root, Elizabeth Town, NC) and a light microscope (Zeiss, New York). A modification of the method was used for counting the cells (10). Briefly, at the end of 48 hours all the contents from the flask were removed and the cells were washed with DPBS once for one minute. DPBS was removed and 1 ml of trypsin was added to the flask. The flasks were then placed in an incubator at 37°C for two minutes. The flask was gently shaken and the cells were detached with the help of a cell scraper. An amount of 20 µl of this solution was taken and added to a hemacytometer. Cells were counted in each of the four grids and the average of that was taken. The final result would be an average number of 10⁴ cells per ml.

Statistical analysis. Data obtained were analyzed using descriptive statistics and single-factor analysis of variance (ANOVA), and were presented as mean value ± S.D.

RESULTS AND DISCUSSION

Cosmetic and personal care products are used on a daily basis, either in the forms of a “wash off,” such as soaps and shampoos, or a “leave on,” such as creams, lotions, and makeup preparations. Over a lifetime, a person living in the Western world is expected to be exposed to thousands of different chemicals repeatedly. While most of these chemicals will not penetrate healthy intact skin, depending on their chemical and physical properties, numerous compounds can partition into the skin and either accumulate in its viable layers or further penetrate to the blood circulation. If these compounds carry toxic potential, their accumulation can lead to diseases and disorders. Moreover, our skin and our internal organs contain metabolic systems that can convert an inert compound into a toxic substance.

Since, unlike pharmaceutical grade compounds, the production of a cosmetic ingredient does not require strict conditions such as GMP, the residuals and the impurities that these ingredients may contain are of major concern. For example, it was shown that polyethylene glycol (PEG) molecules with a molecular weight of around 200 Da can induce a genotoxic effect in chinese hamster ovarian (CHO) cells after metabolic activation with S9 following chromosome aberration study protocols (11). The researchers also concluded that their findings may reflect a potential mutagenic risk of PEG derivatives with similar molecular sizes. Another example is sodium benzoate, which is used extensively as a preservative in cosmetic formulations. In a report issued by the EU Commission Scientific Committee, the SCCNFP, in 2002 (12), the Committee reviewed genotoxicity data generated on sodium benzoate. This compound demonstrated positive results for genotoxicity in a CHO cell line without metabolic activation. Although chromosomal aberrations were not induced when tested *in vivo* in rats, a dominant lethal assay with sodium benzoate in rats did show a genotoxic effect. Based on these data, the committee concluded that sodium benzoate carries a potential genotoxic risk and called for the generation of additional studies to either confirm these observations or rule them out.

Structural aberrations may be of the chromosome or chromatid type. The induction of generation of two or more homologous sets of chromosomes, called polyploidy, may be an indication that a substance carries the potential to induce numerous aberrations that can lead to the initiation of mutations that transform the cells to the cancerous stage. The identification of aberrations in this study is normally conducted by observational assessment of the morphological changes in the nucleus. It therefore requires an experienced individual who is capable of differentiating between a state that is unusual and a state wherein the cell was arrested in the course of normal division. Therefore, we also added quantification of the data to corroborate the microscope observations.

MICRONUCLEI FORMATION

The *in vitro* micronucleus assay is a mutagen test system for the detection of the chemically induced formation of small membrane-bound DNA fragments, i.e., micronuclei in the cytoplasm of cells (13,14). These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division. The micronucleus assay is widely used for monitoring genetic damage caused by different substances. Typical images of cells with stained nuclei and quantitative analysis of micronuclei in cell populations are presented in Figures 2 and 3, respectively. It can be clearly seen that both positive controls induced substantial genetic damage leading to the formation of 140–170 micronuclei per 1000 cells. In contrast, cellular nuclei appear intact after the incubation with OB at both test concentrations and the average number of micronuclei per 1000 cells was close to that of the control cells.

CELLULAR VIABILITY AND PROLIFERATION

A cell that had gone through mutational changes can either go into senescence or apoptosis, or it may survive. If it survives and mutations are not corrected by DNA repair enzymes, these mutations can possibly lead to unregulated cell division and the creation of cancerous tissue. Cancerous cells, therefore, do not obey the normal apoptotic paths that are typical of normal cells. In fact, in cancer cells activation of biochemical substances such as cytokines and other mediators may enhance cell proliferation and viability markers may increase as the cell becomes more sensitive in response to promoters that are involved in the induction of cell division. In addition, in these cells metabolic activity may be accelerated. The MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) test detects the activity of mitochondrial enzymes in viable cells and is an indication of cell viability (15). We tested cell viability in an attempt to draw additional data that will further validate our findings related to chromosomal changes (Figure 4) It was previously shown that a positive cell viability assay response has a strong probability of predicting carcinogenicity *in vivo* (16). In other words, if a compound (such as the positive controls in this study) is generating both micronuclei formation and accelerating cell proliferation and/or metabolism, there are two related pieces of evidence that point to its potential of being a carcinogen. The cell-counting studies also show that the proliferation of the cells incubated with the studied compound are similar to the proliferation of the cells incubated with fresh media (Figure 5).

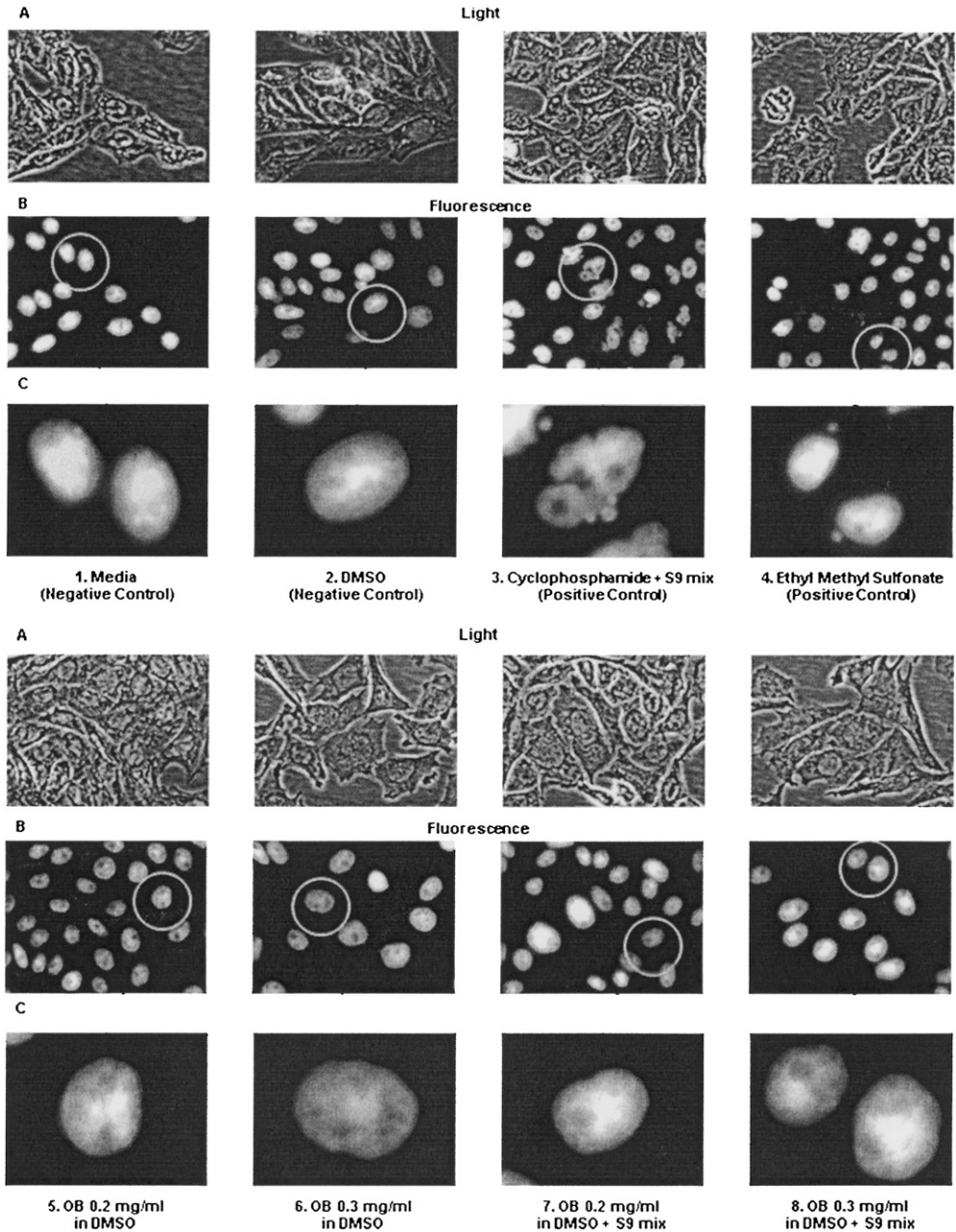


Figure 2. Typical light and fluorescent images of CHO-K1 cells incubated for 24 hours with the following substances: (1) Media (negative control); (2) DMSO (negative control); (3) Cyclophosphamide + S9 mix (positive control); (4) Ethyl methyl sulfonate (positive control); (5) OB, 0.2 mg/ml in DMSO; (6) OB, 0.3 mg/ml in DMSO; (7) OB, 0.2 mg/ml in DMSO + S9 mix; and (8) OB, 0.3 mg/ml in DMSO + S9 mix. The cells were stained with DAPI nuclear dye.

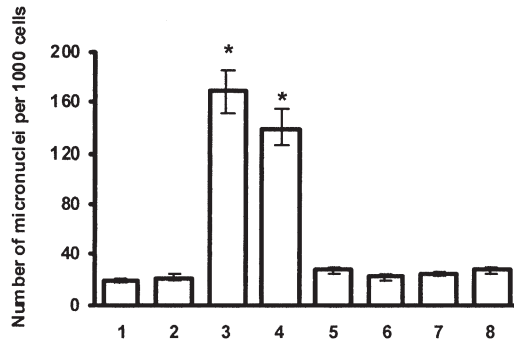


Figure 3. Number of micronuclei per 1000 cells. CHO-K1 cells were incubated for 24 hours with the following substances: (1) Media (negative control); (2) DMSO (negative control); (3) Cyclophosphamide + S9 mix (positive control); (4) Ethyl methanesulfonate (positive control); (5) OB, 0.2 mg/ml in DMSO; (6) OB, 0.3 mg/ml in DMSO; (7) OB, 0.2 mg/ml in DMSO + S9 mix; and (8) OB, 0.3 mg/ml in DMSO + S9 mix. Means \pm S.D. are shown. * $P < 0.05$ when compared with negative control.

CONCLUSIONS AND FUTURE ASPECTS

Under the conditions of the study, the test substance, OB, did not show promotion of the generation of micronuclei with and without enzymatic activation. The measurements of cellular viability showed that cyclophosphamide + S9 mix (10 μ g/ml), ethyl methanesulfonate (EMS, 400 μ g/ml), or OB (0.2 mg/ml and 0.3 mg/ml) alone or in combination with S9 did not inhibit the growth of cells (Figure 4) at the concentrations used. Consequently, we conclude that all tested substances do not promote cell death at the concentrations used and that the positive controls may have generated a shift in cellular function that led to enhanced proliferation.

While this method identifies the genotoxic potential of the tested compounds, there are additional end points that can be studied to better assess the effect of the compounds tested on cell survival. Such is the characterization of cell death by either apoptosis or necrosis.

In addition, this study demonstrates a way to test a compound that is not fully solubilized in the media. Stages of introduction of the OB to the cell culture included dissolution in

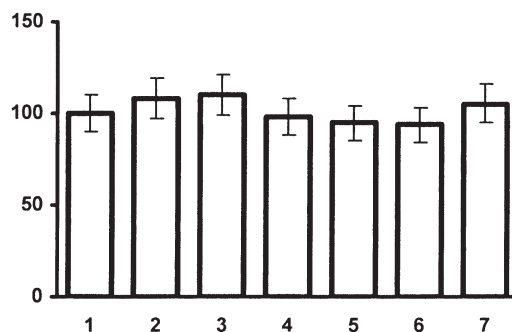


Figure 4. Cellular viability. CHO-K1 cells were incubated for 24 hours with the following substances: (1) DMSO (negative control); (2) Cyclophosphamide + S9 mix (positive control); (3) Ethyl methanesulfonate (positive control); (4) OB, 0.2 mg/ml in DMSO; (5) OB, 0.3 mg/ml in DMSO; (6) OB, 0.2 mg/ml in DMSO + S9 mix; and (7) OB, 0.3 mg/ml in DMSO + S9 mix. Means \pm S.D. are shown (1).

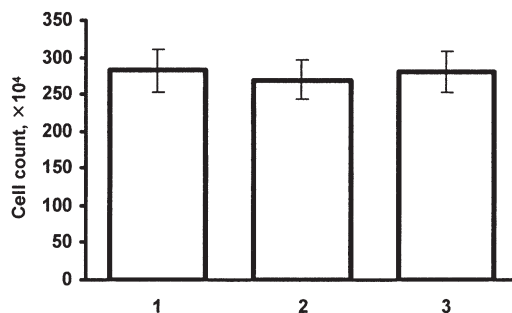


Figure 5. Influence of test substances on cellular proliferation. The same number of cells (3×10^4) were seeded in the flask and incubated with media (1) and a test substance in two concentrations: 0.2 mg/ml (2) and 0.3 mg/ml (3). Cells were counted 48 h after the addition of the test substances. Means \pm S.D. are shown from four independent measurements.

DMSO (which had no effect on the cells when tested alone), sonication, centrifugation, and drawing the supernatant. This is to mimic real-life exposure wherein the particle does not penetrate healthy intact skin, but its impurities and residuals can dissolve in skin fluids.

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