Embryotoxicity estimation of commonly used compounds with embryonic stem cell test

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Abstract. The embryonic stem cell test (EST), an alternative model to animal studies, is a reliable and scientifically validated *in vitro* system for testing embryotoxicity. In contrast to most *in vivo* animal tests, two permanent cell lines, murine fibroblasts (BALB/c-3T3 cells) and murine embryonic stem cells (mES-D3 cells), are used in EST instead of animals in standard tests of toxicity. The embryotoxic potential of compounds (non, weak or strong embryotoxicity) may be obtained with a biostatistics-based prediction model and calculated from three different experimental endpoint values: The potency to inhibit growth of i) BALB/c-3T3 cells and ii) mES-D3 cells (IC_{50}3T3 and IC_{50}ES) as presented using a cell cytotoxicity assay, and iii) the potency to inhibit differentiation of mES-D3 cells into contracting cardiomyocytes (ID_{50} D3) as demonstrated in a mES-D3 cell differentiation assay. In the present study, a model of EST with mES-D3 cells and BALB/c-3T3 cells was established, according to the standard EST system of the EU Center for the Validation of Alternative Methods, and verified it with 5-fluorouracil (strong embryotoxicity) as a positive control and penicillin G (non-embryotoxic) as a negative control. In addition, the authors further assessed the embryotoxicity of four compounds (eugenol, carnosic acid, procyanidin and dioctyl phthalate) with this model. The embryotoxic potentials of the four compounds were successfully classified by the EST system. Eugenol exhibited strong embryotoxicity, carnosic acid and dioctyl phthalate exhibited weak embryotoxicity, while procyanidin exhibited non-embryotoxicity.

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Introduction

The embryonic stem cell test (EST), which was developed by Scholz *et al* (1), was designed for the *in vitro* embryotoxicity testing of drugs and other chemicals (2-6). It uses two permanent murine cell lines: The murine embryonic stem cell line (mES-D3 cells, applied to analyze the effects of compounds on the developing embryo) and the differentiated fibroblast cell line (BALB/c-3T3 cells, applied to analyze the effects of compounds on adult tissues and organs). *In vitro*, murine embryonic stem (mES) cells may be cultured by the hanging drop-suspension-adherence method to form embryoid bodies (EBs) in the absence of anti-differentiation agents (e.g., embryonic fibroblasts and leukemia inhibitory factor, LIF). These EBs, when subsequently seeded in dishes, can spontaneously differentiate to form contracting cardiomyocytes (7-9). The process of differentiation of mES cells into cells of all three germ layers within EBs, as well as the expression of tissue specific proteins, closely resemble the *in vivo* processes in developing embryos (10,11). Therefore treating mES cells during *in vitro* differentiation with the compound of interest may be very useful to obviate unwanted negative effects on embryonic development. The myosin heavy chain (MHC) gene is characteristic of atrial and ventricular cells during early embryonic heart development, and can serve as a marker gene for cardiac development during mES cell differentiation (12). The established EST takes advantage of these properties of mES cells by assessing the degree of inhibition that the test compounds causes in their differentiation processes (13-15).

For classification of the embryotoxic potential of test compounds, three different endpoints could be detected following treatment with the compounds: Cytotoxicity analysis of i) mES cells and ii) 3T3 cells (the concentration of the test compounds resulting in a 50% decrease in the viability of mES cells and 3T3 cells, IC_{50}ES/IC_{50}3T3) and iii) the inhibition of differentiation of mES cells (the concentration of the test compounds that causes a 50% inhibition of the differentiation of mES cells into contracting cardiomyocytes, ID_{50}D3) (16-18).

When the IC_{50} and ID_{50} values are applied to a biostatistical prediction model (PM) developed by the Center for Documentation and Evaluation of Alternative Methods to Animal Experiments and based on linear discriminant functions, the test compounds can be classified into three different classes according to *in vivo* embryotoxic potencies: Strong, weak or non-embryotoxicity (19).

The present study was conducted in two consecutive stages:

Phase I: According to the standard EST protocol of the European Centre for the Validation of Alternative Methods (ECVAM), an EST model was established, and two chemicals with known *in vivo* embryotoxic potential were tested: 5-fluorouracil (strong embryotoxicity) and penicillin G (non-embryotoxic). This was conducted in order to evaluate the feasibility of the model (2,20).

Phase II: The embryotoxicity of four compounds was assessed (eugenol, carnosic acid, procyanidin and dioctyl phthalate) with the EST model.

Eugenol is a biologically active phenolic component of *Syzigium aromaticum* (cloves). It is commonly used in perfumes, flavorings, essential oils and in medicine, due to its various biological properties such as antifungal properties and antioxidation (21-23). Carnosic acid is a phenolic diterpene compound present in considerable quantities in sage and rosemary (24,25). It is increasingly used in food and cosmetic production, as well as in medicine (26-30). Procyanidin is polyphenolic bioactive compound that can be identified in high concentrations in many foods, including grapes, apples and vegetables (31,32). It is also commonly used in drugs, cosmetics and foods (33,34). Dioctyl phthalate, also known as diethylhexyl phthalate, is frequently used as plasticizers in the manufacture of polyvinyl chloride, which is widely used for the production of bags, storage containers and wall coverings, as well as use in medical devices (35-37). In conclusion, these four compounds possess a wide spectrum of applications, and they appear in a wide range of consumer products, as well as in medical applications. People are exposed daily to these compounds through ingestion, inhalation and dermal contact (21,29,33,38). The inclusion of these compounds in personal care or consumer products used by pregnant women should be particularly noted because of the vulnerability of this population; it is crucial to investigate the embryotoxic potential of these compounds.

Materials and methods

Cell culture. mES-D3 cells (CRL1934; ATCC, Manassas, VA, USA) and BALB/c 3T3 cells (CCL-163; ATCC) were cultured at 37˚C in a 5% CO_2 atmosphere. mES cells were routinely cultured on mouse embryonic fibroblast feeder (0303-200; Innovative Cellular Therapeutics, Co., Ltd., Shanghai, China) in the presence of leukemia inhibitory factor (LIF; PMC9484; 1,000 U/ml, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to maintain their undifferentiated status and were passaged every second day. mES cell medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM GlutaMAX-I Supplement, 1% non-essential amino acids (all Gibco; Thermo Fisher Scientific, Inc.), 0.1% β-mercaptoethanol (Merck KGaA, Darmstadt, Germany), 50 U/ml penicillin and 50 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA). Maintenance of BALB/c 3T3 cells used products from Gibco; Thermo Fisher Scientific, Inc., unless otherwise stated. The cells were maintained in DMEM containing 10% FBS, 4 mM GlutaMAX-I Supplement, 50 U/ml penicillin G and 50 μg/ml streptomycin (Sigma-Aldrich; Merck MGaA).

Tested compounds. 5-fluorouracil (CAS no. 51-21-8) and penicillin G (CAS no. 69-57-8), purchased from Sigma-Aldrich; Merck KGaA, were dissolved in 1xPBS or DMEM. As for Phase II chemicals, eugenol (CAS no. 97-53-0) was purchased from Alfa Aesar; Thermo Fisher Scientific, Inc. Carnosic acid (CAS no. 3650-09-7) and procyanidin (CAS no. 4852-22-6) were purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China) and dioctyl phthalate (CAS no. 117-81-7) was purchased from Sigma-Aldrich; Merck KGaA. Eugenol, carnosic acid and dioctyl phthalate were solved in <0.5% ethanol. Procyanidin was dissolved in DMEM.

Assessment of cytotoxicity. The cytotoxic effects of selected test compounds on 3T3 cells and mES-D3 cells were determined with MTT cytotoxicity assay (1). A total of 500 cells in 50 μl routine culture medium without LIF were seeded into each well of a 96 well plate (density, $1x10^4$ cells/ml). Following 2 h incubation at 37˚C in 5% CO_2, culture medium (150 μl), in the presence of the test compound at a range of concentrations, were added into each well except for those for the solvent control and positive control. In Phase I, eight concentrations were set for 5-fluorouracil in 1:10 dilutions from 1 mg/ml and for penicillin G from 10 mg/ml. In Phase II, 5-fluorouracil served as a positive control and the appropriate concentration was set as 0.08 μg/ml for ES cells and 0.25 μg/ml for 3T3 cells. A series of seven concentrations was set for each compound in 1:10 dilutions, from 1 mg/ml. The test substance and the positive control were tested in six independent experiments. The medium was replaced on day 3 and 5 of culture with new medium containing the appropriate concentration of the test chemical. The viability of the cells was determined using an MTT assay. Following 10 days of culture, the medium was replaced with 0.5 mg/ml MTT, and incubated at 37˚C in 5% CO_2 atmosphere for 2 h. Subsequently, MTT medium was removed from the cells. Formazan was extracted from the cells with 100 μl dimethyl sulfoxide (DMSO, Sigma-Aldrich; Merck KGaA) per well. Following agitating the plates on a shaking incubator with a rotational radius of 10 cm at 200 rpm for 15 min, the optical density (OD) value of each well was measured at a wavelength of 570 nm using 630 nm as the reference wavelength in a PARADIGM Detection Platform (Beckman Coulter Inc., Brea, CA, USA), performed according to manufacturer's protocols. The concentration inhibiting 50% viability of ES cells (IC_{50} ES) or 3T3 fibroblasts (IC_{50} 3T3) compared with time-matched solvent treated cells (The OD value of solvent control was set as 100%) was assessed graphically from the corresponding concentration-response curves. The cytotoxicity assay of each compound was repeated three times. The mean IC_{50} value of three repeats was set as the result.

Differentiation of ES cells. As previously described, when undifferentiated mES cells are incubated *in vitro*

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene name	Forward primer sequence	Reverse primer sequence
α/β-MHC	CTTGTTGACCTGGGACTCGG	ACCTGTCCAAGTTCCGCAAG
GAPDH	GCCTTCTCCATGGTGGTGAA	GCACAGTCAAGGCCGAGAAT

MHC, myosin heavy chain.

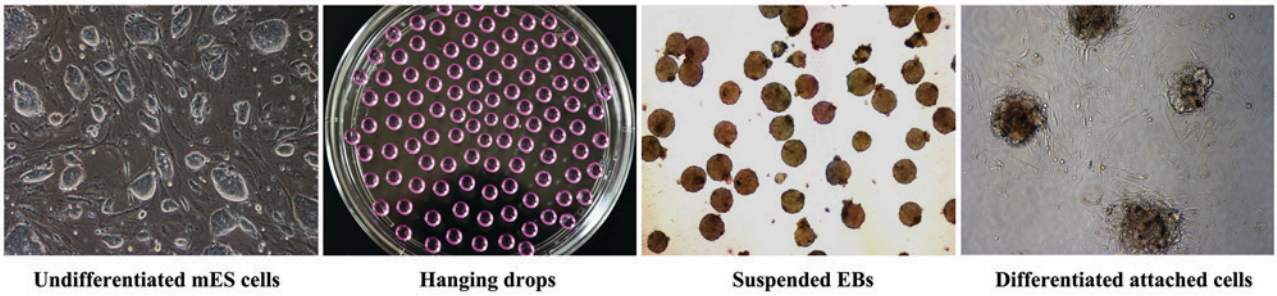

Figure 1. Protocols for mES cell differentiation. Undifferentiated mES cells were cultured using the hanging drop-suspension-adherence method to form EBs. mES, murine embryonic stem cells; EBs, embryoid bodies.

by the hanging drop-suspension-adherence method in ES medium without LIF, the cells can combine to form EBs (Fig. 1) (2,9,15). In brief, ~1,000 mES cells in 20 μl droplets of cell suspension were placed onto the inner side of the lid of a 10 cm Petri dish (BD Biosciences, Franklin Lakes, NJ, USA) filled with 5 ml PBS and then incubated at 37˚C in 5% CO_2 atmosphere. This 'hanging drop' culture was maintained in the absence of LIF to form EBs. Following culturing for 3 days, these EBs were transferred into sterile Petri dishes and cultured in suspension in 5 ml differentiation medium for 2 days. Subsequently, these EBs were seeded on 0.1% gelatin-coated 6-well plates at a density of 100 EBs per well and incubated for an additional 5 days for differentiation into beating cardiomyocytes. mES cells were exposed to the test compound in appropriate concentrations from day 0 onwards over the complete culture duration as described. In Phase I, five concentrations were set for 5-fluorouracil (0.02, 0.04, 0.06, 0.08 and 0.1 μg/ml) and penicillin G (200, 400, 600, 800 and 1,000 μg/ml). In phase II, 5-fluorouracil served as positive control, and the appropriate concentration was set as 0.037 μg/ml. In addition, five concentrations were set for eugenol and carnosic acid (2, 4, 6, 8 and 10 μg/ml), as well as for procyanidin and dioctyl phthalate (20, 40, 60, 80 and 100 μg/ml). The setting of the concentrations of each test compound for differentiation assay was based on the results of the cytotoxicity assay and preliminary experiments. Untreated controls and the corresponding solvent controls were included in each experiment.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis. On day 10, ~100 EBs per sample were harvested, and total RNA was isolated with TRIzol reagent (15596-026; Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized from 1,000 ng RNA per reaction with the Revert Aid First Strand cDNA Synthesis kit (K1622; Thermo Fisher Scientific, Inc.). The α/β-MHC gene expressed specially in cardiomyocyte differentiation was chosen as a marker gene, and GAPDH was chosen as the housekeeping gene. The polymerase chain reaction was performed in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with specific primers (Table I) by initial denaturation at 94˚C for 3 min, followed by 32 cycles of PCR amplification: Denaturation at 94˚C for 30 sec, annealing at 62˚C (α/β-MHC) or 56˚C (GAPDH) for 30 sec, and completed by a final extension of 72˚C for 5 min (16). PCR fragments were run on a 3% agarose gel containing 0.2 μg/ml ethidium bromide (Sangon Biotech Co., Ltd., Shanghai, China), visualized under UV light with a Molecular Imager ChemiDoc XRS system (Bio-Rad Laboratories, Inc.) and analyzed with Quantity One 1-D analysis software version, 4.6.2 (Bio-Rad Laboratories, Inc.) (39). The ID_{50} of the test compound, expressed as the concentration that suppressed the expression of MHC by 50% in comparison with control, was calculated from a concentration-response curve. The differentiation assay of each compound was repeated three times. The mean ID_{50} value of three times was set as the result.

Classification of the embryotoxicity. The embryotoxic potential of each test compound was classified into three grades (strong, weak and non-embryotoxic) based on three values (IC_{50}3T3, IC_{50}ES and ID_{50}), according to the PM proposed by ECVAM (2,19). The values were as follows: I, 5.9157 lg (IC_{50}3T3)+3.500 lg (IC_{50}ES)-5.307 [(IC_{50}3T3-ID_{50}D3)/IC_{50}3T3]-15.72; II, 3.651 lg (IC_{50}3T3)+2.394 lg (IC_{50}ES)-2.033 [(IC_{50}3T3-ID_{50}D3)/IC_{50}3T3]-6.8; and III, -0.125 lg (IC_{50} 3T3)+1.917 lg (IC_{50}ES)+1.500 [(IC_{50}3T3-ID_{50}D3)/IC_{50}3T3]-2.67. The grades were classified as follows: Class 1, non-embryotoxicity, If I>II and I>III; Class 2, weak embryotoxicity, If II>I and II>III; and Class 3, strong embryotoxicity, If III>I and III>II.

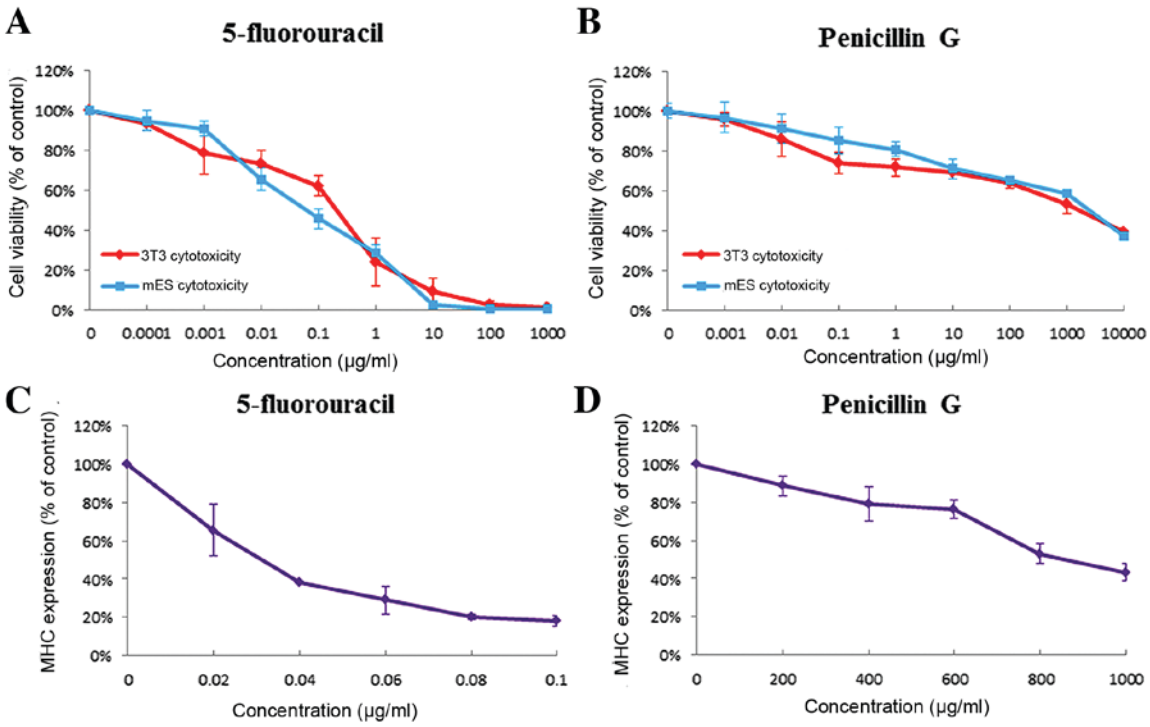

Figure 2. Concentration-response curves of the test compounds: 5-fluorouracil and penicillin G. There were three endpoints of the EST: (A and B) Cytotoxicity of mES-D3 and 3T3 cells and (C and D) inhibition of differentiation of mES-D3 cells were measured for selected concentrations and normalized to control. Inhibition of differentiation was demonstrated by measuring MHC gene expression, as it is a marker of cardiac development during ES cell differentiation. Data are presented as the mean ± standard error of the mean (n=3). mES, murine embryonic stem cells; 3T3, BALB/c 3T3 cells; EST, embryonic stem cell test; MHC, myosin heavy chain.

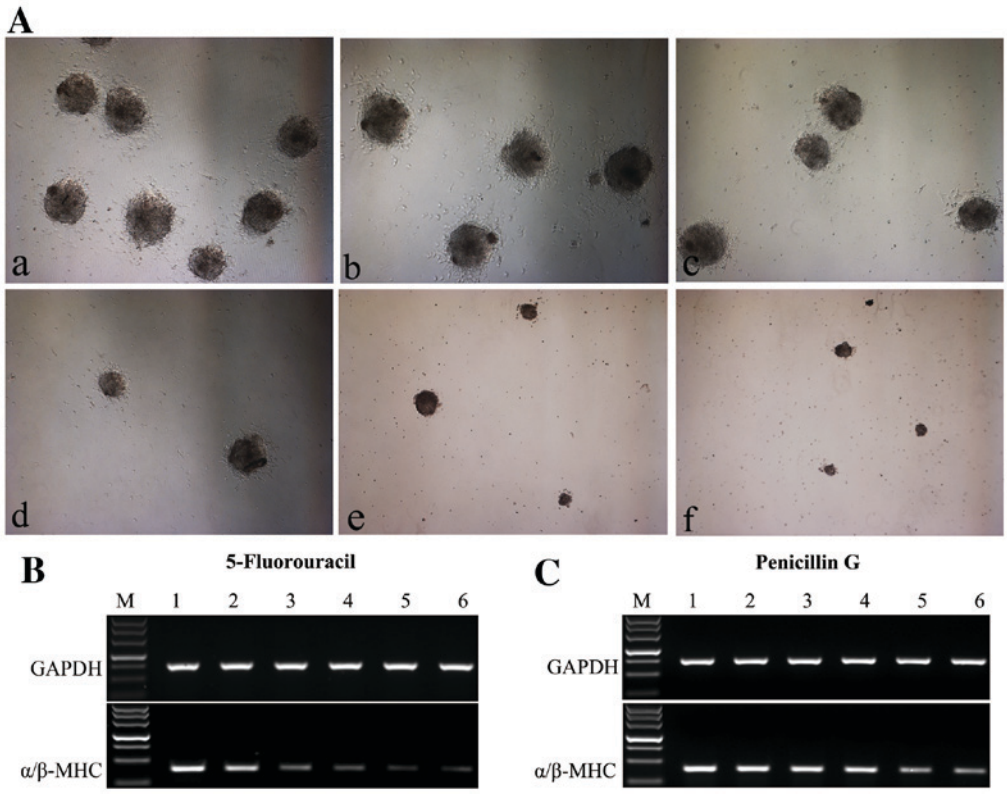

Figure 3. (A) In the differentiation assay, 5-fluorouracil exhibited inhibition for mES cells differentiated into cardiomyoctyes in a dose-dependent manner. a, Solvent control; b, 0.02 μg/ml; c, 0.04 μg/ml; d, 0.06 μg/ml; e, 0.08 μg/ml; f, 0.1 μg/ml. Images were captured at x40 magnification. (B and C) Expression levels of the α/β-MHC in mES cells treated by different concentrations of test compounds were analyzed by reverse transcription quantitative-polymerase chain reaction, normalized to GAPDH. (B) 5-fluorouracil electrophoresis. M, DL500 DNA Marker (500, 400, 300, 200, 150, 100, 50 bp); 1, Solvent control; 2, 0.02 μg/ml; 3, 0.04 μg/ml; 4, 0.06 μg/ml; 5, 0.08 μg/ml; 6, 0.1 μg/ ml. (C) Penicillin G electrophoresis, M, DL500 DNA Marker; 1, Solvent control; 2, 200 μg/ml; 3, 400 μg/ml; 4, 600 μg/ml; 5, 800 μg/ml; 6, 1,000 μg/ml. mES, murine embryonic stem cells; MHC, myosin heavy chain.

Statistical analysis. The statistical analysis was performed using SPSS software (version, 19.0; IBM SPSS, Armonk, NY, USA) Data were expressed as mean ± standard error of the mean. Each data point represented the mean from three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Phase I: Evaluation of the feasibility of the established EST model with control substances. The strongly embryotoxic 5-fluorouracil was used as a positive control and the non-embryotoxic penicillin G was used as a negative control; 5-fluorouracil and penicillin G are the classic positive and negative substances routinely used to evaluate the feasibility of the established EST model (2,5,6,20). In the present study, 5-fluorouracil exhibited strong embryotoxicity. When treated with 5-fluorouracil, the viability of 3T3 cells and mES cells decreased dose-dependently (Fig. 2A) and were influenced at very low concentrations (IC_{50}3T3, 0.244±0.051 μg/ml; IC_{50}ES, 0.080±0.016 μg/ml). In the differentiation assay (Figs. 2C, 3A and B), 5-fluorouracil exhibited strong inhibition of the differentiation of ES cells into contracting cardiomyoctyes (ID_{50}, 0.037±0.006 μg/ml) as indicated by the expression of marker gene (α/β-MHC) analyzed by RT-PCR. Test results demonstrated that penicillin G exhibited little cytotoxicity to 3T3 cells or mES cells (IC_{50}3T3, 1,160.667±69.07 μg/ml; IC_{50}ES, 1,567.497±152.471 μg/ml; Fig. 2B) and weak inhibition of ES cells differentiation into contracting cardiomyoctyes (ID_{50}, 980.098±24.693 μg/ml; Figs. 2D and 3C). Even at the highest concentration tested (1,000 μg/ml), only a minor inhibition was observed. According to the PM, 5-fluorouracil and penicillin G were classified as reagents with strong embryotoxicity and non-embryotoxicity, respectively. The embryotoxicity classifications were the same as those in the ECVAM validation study.

Phase II: Embryotoxicity assessment of four compounds with the model of EST. In order to expand the application of EST, the embryotoxic potentials of four selected compounds (eugenol, carnosic acid, procyanidin and dioctyl phthalate) were assessed with the model in phase II.

Eugenol. Test results demonstrated that eugenol presented strong embryotoxicity in the EST. Both 3T3 cells and mES cells were markedly sensitive to the cytotoxic effect of eugenol (IC_{50}3T3, 9.441±2.849 μg/ml; IC_{50}ES, 1.929±0.329 μg/ml; Fig. 4A). In the differentiation assay (Figs. 4E and 5A), when the EBs were exposed to eugenol, a concentration-dependent inhibition of differentiation was observed. Treatment with eugenol resulted in the direct inhibition of differentiation of mES-D3 cells at very low concentrations (ID_{50}, 5.434±0.715 μg/ml).

Carnosic acid. In the cytotoxicity assay, the test results indicated that carnosic acid exhibited greater cytotoxicity effects on mES cells than on 3T3 cells (IC_{50} 3T3, 26.28±3.861 μg/ml; IC_{50}ES, 5.771±1.297 μg/ml; Fig. 4B). Exposure of the EBs to carnosic acid, inhibition of differentiation into cardiomyocytes was detected at similar concentrations as the IC_{50} test in ES cells (Figs. 4F and 5B) and the ID_{50} was 6.143±0.575 μg/ml.

Procyanidin. As presented in Fig. 4C, 3T3 cells were notably sensitive to the cytotoxic effect of procyanidin in comparison to mES cells (IC_{50}3T3, 12.1±1.828 μg/ml; IC_{50}ES, 145.139±21.121 μg/ml) in the cytotoxicity assay. The differentiation assay demonstrated that procyanidin had weak inhibition of ES cell differentiation (ID_{50}, 72.493±2.706 μg/ml; Figs. 4G and 5C).

Dioctyl phthalate. In the EST, when the cells were treated with dioctyl phthalate, greater cytotoxicity effects were identified on mES cells than on 3T3 cells (IC_{50} 3T3, 213.487±28.158 μg/ml; IC_{50} ES, 123.587±24.944 μg/ml; Fig. 4D). Under dioctyl phthalate treatment, the authors demonstrated that it could inhibit the differentiation of mES cells at relatively low concentrations (ID_{50}, 60.116±5.39 μg/ml; Figs. 4H and 5D).

Subsequently, the mean values of IC_{50}3T3, IC_{50}ES and ID_{50} of each compound were substituted into the PM, and the embryotoxic potentials of each compound were successfully classified: Eugenol displayed strong embryotoxicity, carnosic acid and dioctyl phthalate displayed weak embryotoxicity, while procyanidin displayed non-embryotoxicity. Summary results are presented in Table II.

Discussion

In the early 1960s, >10,000 infants were born with phocomelia (malformation of the limbs) due to exposure to thalidomide throughout the world (40). The negative effects of thalidomide have focused worldwide attention squarely on the embryotoxicity caused by environmental insults (e.g., drugs, diet and environmental toxic chemicals). At present, a wide range of compounds needs to be tested, and, in particular, the development of those compounds that may be used in pregnant women (those with low toxic potency) must be prioritized. Currently, embryotoxicity is mainly detected by *in vivo* tests. However, *in vivo* detection requires a large number of experimental animals and generous amounts of test chemical with long duration, which altogether make the studies extremely costly (41,42). The EST is currently the only test method that is completely free from use of animals. The validation study of EST funded by ECVAM, presented a good overall test accuracy of 78% for classification of the 20 tested chemicals with known *in vivo* embryotoxic potential (43). In particular, the predictability of 100% for strongly embryotoxic chemicals was obtained, and the precision was considered to be fairly good (2,3,43).

In the present study, a model of EST was established according to the standard EST system of ECVAM, and the validity of the model was verified with 5-fluorouracil as a positive control and penicillin G as a negative control. During pregnancy, the developing embryo is very sensitive, and a variety of compounds have been reported to be toxic or teratogenic for its development (44,45). Eugenol, carnosic acid, procyanidin and dioctyl phthalate, commonly-used compounds, have already been used food, cosmetic and medical applications, within a specific range of concentrations. However, little is known about their influence on embryo development. To the best of the authors' knowledge, there are no available studies describing the effect of these compounds on the embryotoxicity *in vitro*. For this reason, subsequently,

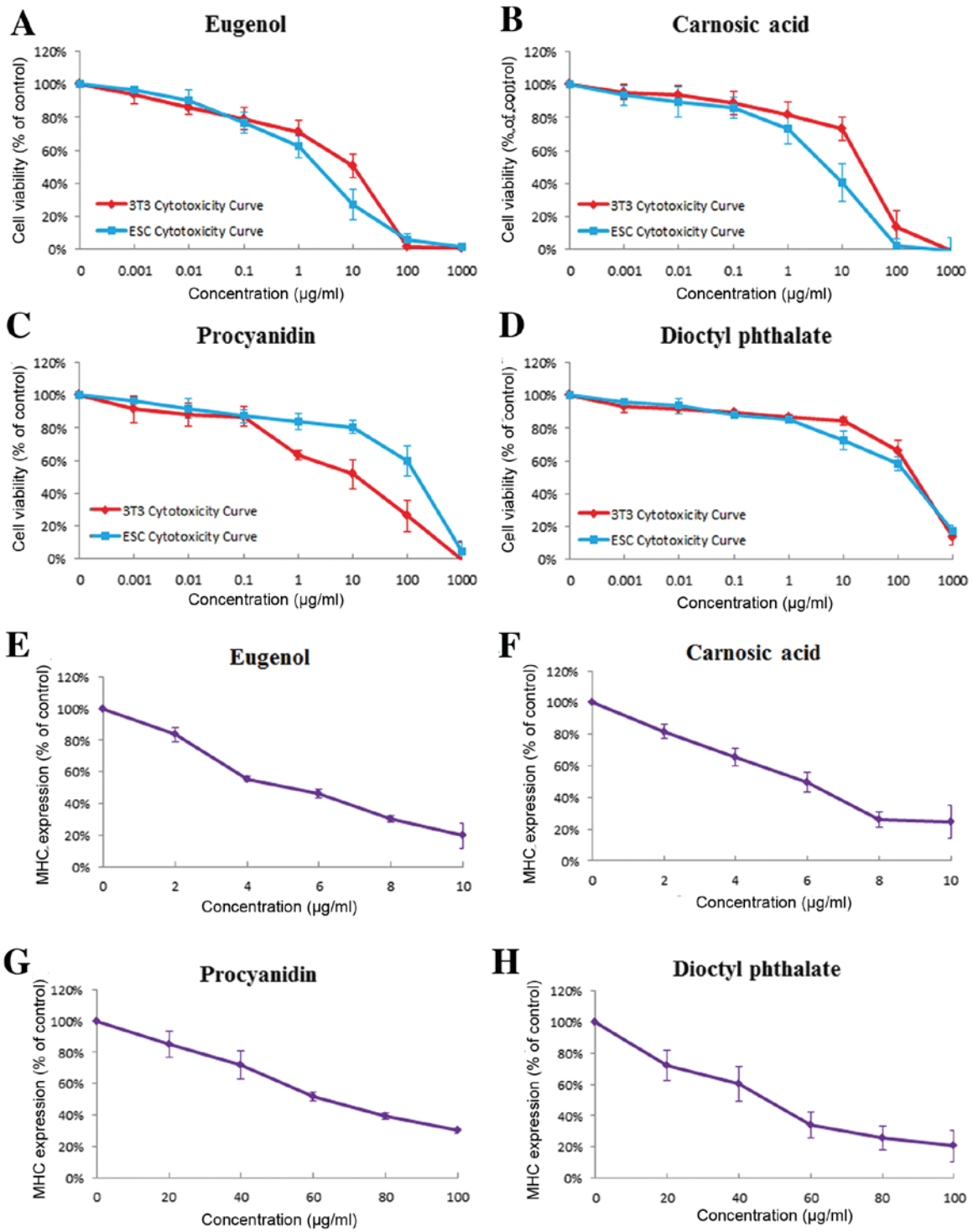

Figure 4. Concentration-response curves of test compounds (eugenol, carnosic acid, procyanidin and dioctyl phthalate). The three endpoints of the EST were tested: (A-D) Cytotoxicity of mES and 3T3 cells and (E-H) inhibition of differentiation of mES cells were measured for selected concentrations and normalized to the control. Data are presented as the mean ± standard error of the mean (n=3). EST, embryonic stem cell test; mES, murine embryonic stem cells; 3T3, BALB/c 3T3 cells; MHC, myosin heavy chain.

the present study assessed the embryotoxicity of these four compounds with the established EST model.

Domaracky *et al* (46) studied the influence of eugenol on the development of mouse preimplantation embryos *in vivo*. The study indicated that eugenol may induce a significantly increased rate of cell death and affect the development of embryo. For its isomer, isoeugenol, when received by pregnant rats (at the highest dose of 1,000 mg/kg/day), it caused intrauterine growth retardation and skeletal defects in fetuses (47). The current results indicated that 3T3 cells and mES cells were both sensitive to eugenol. A higher incidence of cell death was observed following treatment with eugenol. According to the test data, eugenol was classified as strongly embryotoxic. Research on carnosic acid has demonstrated that a short-term

Table II. Summary of mean IC_{50}3T3, IC_{50}ES and ID_{50} values obtained in three independent experiments and classification of test compounds according to the prediction model. Data are presented as IC_{50} and ID_{50} values (μg/ml) ± standard error of the mean, and embryotoxicity was classified as strong, weak or non-embryotoxic.

Tested compound	IC_{50} 3T3 (μg/ml)	IC_{50} ES (μg/ml)	ID_{50} (μg/ml)	Classification of the embryotoxicity
5-fluorouracil	0.244±0.051	0.080±0.016	0.037±0.006	Strong
Penicillin G	1160.667±69.070	1567.497±152.471	980.098±24.693	None
Eugenol	9.441±2.849	1.929±0.329	5.434±0.715	Strong
Carnosic acid	26.280±3.861	5.771±1.297	6.143±0.575	Weak
Procyanidin	12.100±1.828	145.139±21.121	72.493±2.706	None
Dioctyl phthalate	213.487±28.158	123.587±24.944	60.116±5.390	Weak

3T3, BALB/c-3T3 cells; ES, murine embryonic stem cells.

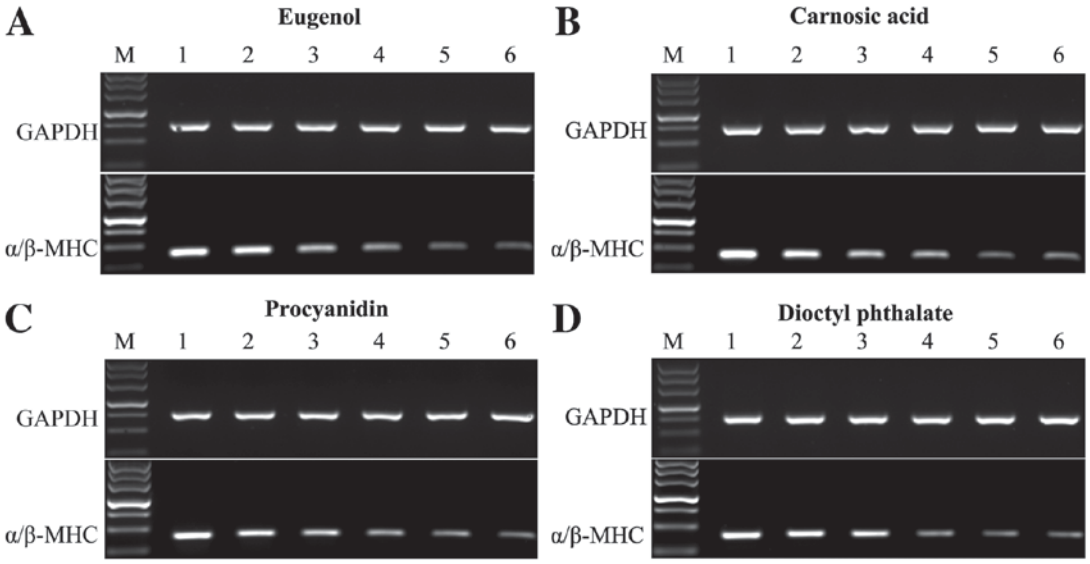

Figure 5. Expression levels of the α/β-MHC at different concentrations of test compounds were analyzed by reverse transcription quantitative-polymerase chain reaction, normalized to GAPDH expression. (A and B): Eugenol, carnosic acid. M, DL500 DNA Marker; 1, Solvent control; 2, 2 μg/ml; 3, 4 μg/ml; 4, 6 μg/ml; 5, 8 μg/ml; 6, 10 μg/ml. (C and D): Procyanidin, dioctyl phthalate. M, DL500 DNA Marker; 1, Solvent control; 2, 20 μg/ml; 3, 40 μg/ml; 4, 60 μg/ml; 5, 80 μg/ml; 6, 100 μg/ml. MHC, myosin heavy chain.

oral administration has a relatively low toxicity profile, and the oral lethal dose for mice was 7,100 mg/kg of body weight (48). The present study suggested that carnosic acid was weakly embryotoxic. Therefore, for the use of carnosic acid, pregnant women must be cautious. However, toxicological studies have indicated that procyanidin is nontoxic and does not cause any detrimental effects *in vivo* (49,50). According to these results, procyanidin is safe and non-embryotoxic under the conditions investigated in the present study. With regards to dioctyl phthalate, many studies on animals clearly demonstrated that dioctyl phthalate could cause a certain tissue/organ toxicity (51-54), developmental toxicity (55-58) and reproductive toxicity (37,59-61) in some species, such as rats, mice and marmosets (36,62,63). To the best of the authors' knowledge, there are no data on the embryotoxicity of dioctyl phthalate obtained by *in vitro* animal-free tests. The present study employed the established EST to predict the embryotoxicity of dioctyl phthalate *in vitro*, and the results indicated that dioctyl phthalate exhibited weak embryotoxicity.

Taken together, the authors successfully established the model of EST, and further assessed embryotoxicity of four selected compounds with this model. In the future, it will be important to determine the embryotoxicity of the many commonly used compounds. The EST system for embryotoxicity screening test is rapid, simple and sensitive. It may be used for high-throughput screening of embryotoxicity of test substance.

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