Review Article

Development of technique for *in vitro* embryotoxicity of dental biomaterials

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**KEYWORDS**

EST; Embryotoxicity; Dental materials; ES-D3 cells; Hepatocyte

**Summary** The Embryonic Stem Cell Test (EST) developed in Germany in 1997 is known as a screening test method capable of predicting the presence of unknown chemicals influencing normal human development. Firstly, we investigated the embryotoxicity of 24 types of monomer including dental monomers and dental alloy-component metal elements using this test. Monomers including Bis-GMA contained in base resin of composite resin exhibited weak embryotoxicity, and the toxicity level varied among dental alloy-component metal elements. It was clarified that metal ions eluted from currently sold dental alloys show no embryotoxicity. Then, we investigated a method that also considers human metabolic activity, which is not possible with the EST, in the results of embryotoxicity. In addition, an evaluation method using a hybrid culture system for hepatocytes and mouse ES cells and a method using oviduct or uterus cells for feeder cells were also investigated.

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1. In vitro embryotoxicity test method

The influence of chemical substances on human fetal development is of marked concern. Thalidomide [1,2] is a chemical substance known to influence human fetuses, but the presence of as yet unknown toxic chemical substances among those whose influence has not been fully confirmed cannot be ruled out. The embryotoxicity lacks clinical data, unlike general cytotoxicity, and systematic elucidation remains insufficient. Thus, it is possible that novel synthetic chemical or natural substances show toxicity. Only experimental methods using animals were previously available to assess embryotoxicity, and no in vitro screening method has been fully established.

The Embryonic Stem Cell Test (EST) developed in Germany in 1997 [3] is an in vitro screening method capable of predicting the embryotoxic risk of chemical substances contained in dental materials and drugs. It is an in vitro embryotoxicity test method using 3 parameters: the differentiation and viability of mouse-derived ES-D3 cells and viability of Bulb/c 3T3 cells. The 3 parameters are applied to equations of improved Prediction Model (iPM, Fig. 1), and the test substance is classified into 3 categories: "strongly", "weakly", and "non-embryotoxic". The ES-D3 and Bulb/c 3T3 cells used are shown in Fig. 2, and each culture medium is shown in Table 1. International validation of the EST has been performed in Europe by comparison with other in vitro embryotoxicity test methods (micro mass culture and whole embryo culture methods) [4–6].

In European REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) aimed at re-evaluation of the safety of chemical substances, biological safety evaluation is required for all chemical substances already sold in Europe, including novel synthetic and natural chemical substances. If these chemical substances can be evaluated in vitro without using an experimental animal, it would be advantageous economically, temporally, and statistically, and REACH also recommends it from the viewpoint of animal welfare [7–9].

The EST was demonstrated to be more strongly correlated with human teratogenicity compared to the other 2 methods. Using the EST protocol, we investigated various monomers and dental alloy components. Eighteen types of monomer including dental monomers (1.6-ADMA, 1.8-ADMA, 1.10-ADMA, 2.0-EpDMA, 3.0-EpDMA, 4.0-EpDMA, 6-HHMA, Bis-GMA, Bis-MPEPP, BPE-1300, BSNa, EDMABA, GAM, GMR, UDMA, MEPC, MTYA, Phosmer M, PTSNa, QTX and TEGDMA, Fig. 3) were investigated. The 3 parameters were applied to Equations I, II, and III of the iPM to investigate the embryotoxicity risk. 6-HHMA, Bis-GMA, Bis-MA (6F), Bis-MPEPP, BPE-1300, BSNa, EDMABA, GAM, GMR, UDMA, MEPC, MTYA, Phosmer M, PTSNa, QTX and TE GDMA (Registration, comparison) were compared. Twelve dental alloy-component metals: Ag, Co, Cr, Cu, Hg, In, Ni, Pd, Sb, Sn, V, and Zn, were subjected to the embryotoxicity test employing the EST using standard reagents for atomic absorption measurement. Hexavalent Cr and Hg ions were class 3, i.e., "strongly embryotoxic". In, Sb, and V were "weakly embryotoxic", and the other metal ions were "non-embryotoxic". In addition, test samples of Ag, Co, Cr, Ni, and Pd were prepared by direct extraction from each metal powder in medium and subjected to the test. Only Ag was "weakly embryotoxic", showing embryotoxicity, but all other metal ions were "non-embryotoxic". Hexavalent Cr showed strong embryotoxicity, but trivalent Cr was "non-embryotoxic". Currently, these metal ions are not used in dental care [13]. In addition, the elution of metal ions into saliva markedly decreases in the presence of oxide film on the dental alloy surface. Particularly, the rates of elution of alloy-component ions from dental cobalt-chromium and silver alloys into the oral cavity are low due to the presence of an inert film on the alloy surface. Thus, the influence of metal ions eluted from these dental alloys on embryotoxicity may be negligible.

2. Modification of EST protocol

The embryotoxicity level of mercury varied depending on the ion extraction conditions. The strong embryotoxicity of Hg ions was clarified, but dentists and patients may not be exposed to Hg because amalgam is no longer used in dental care. However, amalgam remains in the oral cavity mainly in elderly patients, and mercury vapor from amalgam present in their teeth is of concern. Mercury vapor markedly
influences human health, unlike metal mercury. To develop an in vitro embryotoxicity test method for mercury vapor, we investigated the application of the roller tube culture method, in which a large culture bottle containing mercury vapor generated by heating metal mercury and medium was slowly rotated to directly expose cells to the phase containing mercury vapor (Fig. 4). The results on ES cells exposing to mercury vapor were different from those on exposure to metal mercury. A very strong influence was noted leading to the impairment of beating which develops through
Table 2  Embryotoxicity risk of monomers.

| Embryotoxicity level | Monomers                                                                 |
|----------------------|--------------------------------------------------------------------------|
| Strong (III)         | None                                                                     |
| Weak (II)            | 6-HHMA, Bis-GMA, Bis-GMA(6F), MTYA, Bis-MPEPP, TEGDMA, UDMA            |
| None (I)             | 1,6-ADMA, 1,8-ADMA, 1,10-ADMA, 2.0 EpDMA, 3.0 EpDMA, 4.0 EpDMA, BPE-1300, BSNa, GMA, GAM, EDMABA, Phosmer M, PTSNa, QTX |

* These monomers are boundary area between I.

Figure 4  Exposure to mercury vapor using the rotary culture bottle. We constructed a heat-driven mercury evaporator consisting of a bottle equipped with a 100-V built-in electric heater on the lid and a stainless-steel rod with a slight dent at the tip. The lid of the culture bottle was removed after a rotary culture, and the mercury evaporator with 5.0 μg or 2.0 μg of mercury at the tip of the stainless-steel rod was placed inside the bottle. Mercury evaporated completely after the electricity was turned on and the stainless-steel rod was heated for 10 min. After changing the lid, a rotary culture was done at 37 °C for three days in an incubator (New Brunswick).

Figure 5  (a) Results of the EBs differentiation in mercury vapour. The mercury exposure concentrations were 5.0 μg and 2.0 μg, no cell pulsations were observed, whereas in the control group with no mercury exposure, myocardial cell pulsations were observed in 84.0% of the wells. (b) Results of cell viability in mercury vapour with MTT method. The mercury exposure concentration was 5.0 μg, the cell viability was 68% of that of the control group. Also, in the group where the mercury exposure concentration was 2.0 μg, the cell viability was 96.0% of that of the negative control group.
Figure 6  (a) Three-dimensional cell culture. Collagen gel or sponge was placed in cell culture inserts, and 300 μL of medium was poured into the cell culture insert, with 1.6 mL of medium being poured in the wells of a 12-well multi-dish. (b) Osteocalcin levels. After 21 days of culture, the levels of the bone differentiation marker mouse osteocalcin were measured by ELISA assay kit (Biomedical Technologies, Inc., USA). The mean osteocalcin value was 2.139 for CG and 1.664 for CS, whereas the level detected for FC was not significantly different from that observed for the negative control group. (c) A collagen gel (Nitta Gelatin, Osaka) derived from porcine (Type I-A and Type III collagens mixed in a 3:1 ratio by volume; CG), a collagen gel derived from chum salmon (Ihara & Co., Ltd., Rumoi, Hokkaido, Type I; FC) and a collagen sponge derived from porcine (Nitta Gelatin, Type I; CS) were placed separately in 10 mm-diameter cell culture inserts (Falcon, USA) and fixed onto a membrane filter at the bottom of each insert (Gibco). Then, 300 μL of medium was poured into the cell culture insert, with 1.6 mL of medium being poured in the 12-well multi-dish (Falcon). The dishes were cultured in a CO₂ incubator for 24 h. Two EBs per well were placed on a scaffold and cultured in a CO₂ incubator for up to 21 days. The cells were photographed under an inverted phase-contrast microscope (Olympus, Tokyo) after 4, 10, and 21 days of culture. In CG and CS, after 21 days of culture, numerous oval structures and needle-like structures, respectively, were observed, whereas no such structures were observed in FC.
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differentiation from EB to myocardial cells (Fig. 5). Although the experimental conditions, such as exposure time, were different from those in the test with metal mercury, mercury vapor showed strong embryotoxicity [14].

Chemical substances enter the human body through various routes in vivo. Only one chemical substance can be tested each time using the EST, but many chemical substances are mixed in most dental materials and drugs. Thus, it is necessary to assess the embryotoxicity risk of each individual chemical component and make a judgment based on the overall results. However, risk assessment of mixtures remains to be further investigated because a synergistic effect is observed in general toxicity.

It is a disadvantage of the EST protocol that the influence of human metabolic activity is not reflected because of the use of mouse-derived ES and Bulb/c 3T3 cells. The chemical structure of substances may be changed by metabolism in the liver, and the embryotoxicity level may change in the actual body. In addition, the solubility of chemical substances in culture medium is important, which is common to in vitro cytotoxicity experiments. For water-insoluble chemical substances, the EST protocol specifies dissolving it in a solvent, such as ethanol and DMSO, and then culture medium. However, even though the solvent becomes transparent by stirring and the chemical substance appears to have completely dissolved, the solution becomes milky white and precipitates when it is added to culture medium.

When a substance cannot be completely dissolved, cytotoxicity is likely to be reduced, decreasing the accuracy of evaluation. We prepared a 3-dimensional model by mixing cells in collagen, referring to a commercial 3-dimensional skin model, and developed a method to directly expose it to a chemical substance without the need for a solvent (Fig. 6a–c). Considering the results of the present study, we concluded that collagen derived from salmon cannot be used for differentiation of ES-D3 cells [15,16].

We investigated the influences of thalidomide and dental metal-component elements (Ag, Cu, Pd, and Zn) on embryotoxicity. The culture supernatants of commercial TEST LIVER™-human (Fig. 7a), which can be cultured for a prolonged period while maintaining human liver functions, and mouse ES-D3 cells were mixed, and the contractile rate of heart muscle that had differentiated from EBs prepared by hanging-drop culture of ES-D3 cells was investigated. The heart muscle contractile rate was 66.7% in the groups without the addition of a test sample. Thalidomide has been reported to clinically show strong embryotoxicity (Fig. 7b). The differentiation rate of EBs was significantly decreased by Ag and thalidomide, but it was slightly increased by Cu and Zn. No significant difference due to the addition of Pd was noted (Fig. 7c) [17]. The reduction of the differentiation rate of EBs by thalidomide was smaller in the absence than presence of metabolic activation, suggesting that metabolic activation made the findings more consistent with human clinical data. On the other hand, there are no human clinical data on embryotoxicity with which the influence of metal ions on cell differentiation can be compared. Moreover, all metal elements investigated are frequently used as dental alloy components. Therefore, the further collection of embryotoxicity data and their careful analysis are necessary. Regarding cytotoxicity, the correlation of cell viability with metabolic activation obtained using rat TEST LIVER™ with animal study data is stronger than that of the data without metabolic activation, but more accurate data may be collected by modifying the culture time to introduce metabolic activity and the experimental method.

Aiming at the development of a method to evaluate hepatocytes and mouse ES cells using a hybrid culture system,
we compared 2 types of scaffold between the basic structure of the hybrid culture system and 3-dimensional culture method to prepare a specific hybrid culture system method for 3 types of cultured hepatocyte and mouse ES cells. Human- and mouse liver-derived floating cells and human liver cancer-derived HepG2 cells were enclosed in collagen gel. The 3 types of hepatocytes were placed in the inner cell and EBs were placed in the outer region, or EB was enclosed in collagen and placed in the inter cell and the 3 types of hepatocytes were placed in a 12-well multidish in which the bottom was treated with 0.1% gelatin solution for one hour beforehand (Fig. 8a and b). The contractile development rate was slightly higher when EB was placed in the inter cell [18]. These culture techniques are likely to be referred to in order to develop hybrid culture systems in the future.

Regarding ES cell differentiation, since the original inner cell mass of ES cells interacts with the oviduct or uterus cells in the body, intercellular communication between these and ES cells may be established for normal cell division in the body. Thus, we investigated ES cell differentiation using mouse reproductive organ-derived uterus cells and oviduct-derived primary cells as a feeder layer. When 3 types of uterus-derived cell and oviduct-derived primary cells were used as feeder cells, the results differed among the feeder cell types (Fig. 9). The ES cell differentiation rate decreased on a feeder layer comprised of feeder cells and mixed cells showing atypia in their size and morphology [19].

We are planning to further modify the EST, which is an in vitro embryotoxicity test method. We are also investigating the application of these results to not only ES but also iPS cells.

In Japan, a contract research organization for implementing the biological safety tests as a pre-clinical study. In a few cases, EST is already beginning to be described in the test menu. Furthermore, JaCVAM (Japanese Center for the Validation of Alternative Methods) are beginning to consider the embryonic toxicity screening test (Hand1-Luc) protocol as an establish guideline for new alternative animal test method about the same procedure as EST protocol. Validation of this new protocol will start examined by JaCVAM from February of this year.

Figure 8  (a) Human hepatocytes and human liver cancer HEPG2 cells. Human hepatocytes (HF, Celsis IVT, BioreclamationIVT.com, MD, USA), mouse hepatocytes (MF, Celsis IVT), and human liver cancer HEPG2 cells (HG, ATCC:HB-8065) were used. (b) A two-dimensional culture system was prepared with hepatocytes encapsulated in collagen gel (a mixture of Types I and III (Nitta Gelatin Inc., Osaka)) placed inside the 12-well culture inserts (Becton, Dickinson and Co., NJ, USA) and embryoid bodies (EBs) prepared by the suspension culture of ES-D3 cells placed outside. Conversely, an additional system was prepared with EBs encapsulated in collagen gel placed inside the 12-well culture inserts and hepatocytes seeded onto a 12-well multi-dish bottom treated with a 0.1% gelatin solution (STEMCELL™ Technologies Inc., Canada) for an hour.
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The uterus and oviduct tissues of an 11-week-old DBA/2NCrIcrj mouse were cut into fragments, followed by treatment with a 0.25% trypsin/EDTA solution (Invitrogen, CA, USA). The fragments were centrifuged several times, placed in a 25-mL culture flask with 8 mL of DMEM (Gibco, NY, USA) supplemented with FCS (Hyclone, UT, USA) at a volume rate of 10%, and cultured for seven days in a CO₂ incubator at 37 °C. Cells that reached confluence on the flask bottom after 14-day culture were plated. The flasks were divided into S (1)–(3) and O (1)–(3) for uterine- and oviduct-derived cells, respectively. The cells were plated into a 12-well dish and cultured for 24 h, followed by treatment with mitomycin C (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for two hours. EL M3 cells were purchased from the RIKEN BioResource Center (Tsukuba, Japan). EBs, prepared from the suspension culture of EL M3 cells (mouse ES cells), were carefully placed onto the feeder layer and cultured for seven days. Commercial MEF (ReproCell Inc., Kanagawa, Japan) was used as a control feeder layer. The S (1)–(3) and O (1)–(3) cells at 1 × 10⁴ cells/mL were mixed on ice, followed by the addition of reconstitution buffer (Nitta Gelatin). The contraction rate under thalidomide-free conditions was 66.7% for the MEF. The contraction rates of uterine-derived S (1)–(3) and oviduct-derived O (1)–(3) were 60 and 66.7%, respectively. No significant difference was noted between the uterine- or oviduct-derived cells and the MEF. The oviduct-derived cells showed a slightly lower average value, although some showed higher contraction rates than the MEF.

Figure 9 Comparison of inverted phase contrast microscope images of human hepatocytes used in the feeder cells. The uterus and oviduct tissues of an 11-week-old DBA/2NCrIcrj mouse were cut into fragments, followed by treatment with a 0.25% trypsin/EDTA solution (Invitrogen, CA, USA). The fragments were centrifuged several times, placed in a 25-mL culture flask with 8 mL of DMEM (Gibco, NY, USA) supplemented with FCS (Hyclone, UT, USA) at a volume rate of 10%, and cultured for seven days in a CO₂ incubator at 37 °C. Cells that reached confluence on the flask bottom after 14-day culture were plated. The flasks were divided into S (1)–(3) and O (1)–(3) for uterine- and oviduct-derived cells, respectively. The cells were plated into a 12-well dish and cultured for 24 h, followed by treatment with mitomycin C (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for two hours. EL M3 cells were purchased from the RIKEN BioResource Center (Tsukuba, Japan). EBs, prepared from the suspension culture of EL M3 cells (mouse ES cells), were carefully placed onto the feeder layer and cultured for seven days. Commercial MEF (ReproCell Inc., Kanagawa, Japan) was used as a control feeder layer. The S (1)–(3) and O (1)–(3) cells at 1 × 10⁴ cells/mL were mixed on ice, followed by the addition of reconstitution buffer (Nitta Gelatin). The contraction rate under thalidomide-free conditions was 66.7% for the MEF. The contraction rates of uterine-derived S (1)–(3) and oviduct-derived O (1)–(3) were 60 and 66.7%, respectively. No significant difference was noted between the uterine- or oviduct-derived cells and the MEF. The oviduct-derived cells showed a slightly lower average value, although some showed higher contraction rates than the MEF.
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