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Review Article

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

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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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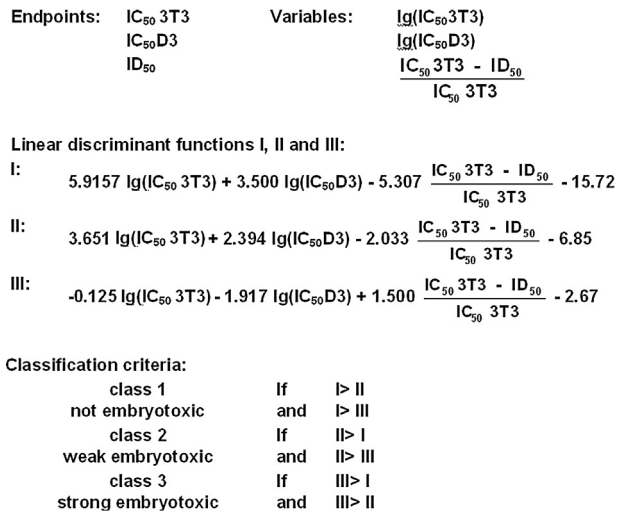

Figure 1 The improved prediction model (iPM). The mathematical formula to predict the embryotoxicity risk level by the three endopoints.

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 embryotoxicity 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].

Table 1 Mediums of both cells.

ES-D3 cells	Balb c/3T3 cells
20% FCS	10% FCS
2 mM L-glutamine	4 mM L-Glutamine
50 IU Penicillin	50 IU Penicillin
50 μg Streptmycin	50 μg Streptmycin
1% NAA	
0.1 mM β-Mercaptoethanol	
1000 U/mL mLIF	

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-GMA (6F), 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-GMA (6F), Bis-MPEPP, MTYA, UDMA, and TEGDMA were Class 2, ''weak embryotoxicity''. The other monomers were Class 1, ''non embryotoxicity'', and no monomer showed ''strong embryotoxicity'' (Table 2). It was clarified that monomers including Bis-GMA contained in base resin of composite resin show weak embryotoxicity [10–12].

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, Sn, 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