ORIGINAL ARTICLE

In Vitro Embryotoxicity Testing of Mercury Vapour by Differentiation of ES-D3 Cells

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Abstract
The embryonic stem cell test (EST) is an in vitro assay that has been developed to assess the embryotoxic potential of chemicals and biomaterials. It is based on the capacity of the mouse embryonic stem cell line D3 (ES-D3 cells) to differentiate into contracting myocardial cells. However, the EST method cannot fully test the embryotoxicity of mercury vapor. We tried to apply the rotary culture bottle containing mercury vapor and culture medium was slowly turned and the cells come into direct contact with the gaseous phase containing mercury vapor. At the mercury exposure concentrations tested in this study, no cell pulsations were observed. The cell viability was affected to some extent in the 5.0μg mercury concentration group, whereas in the 2.0μg group little difference was observed from that of the control group. Although mercury vapor was observed to strongly impair the differentiation of EBs in this study, the test results this time did not prove that mercury vapor has a strong embryotoxic effect on humans. We think it important to develop a device that closely mimics the actual exposure conditions of the human body, including an exposure method that is different from that used in this study, as an alternative to animal experiments.

Key words: mercury vapour, embryotoxicity, ES cell

Introduction
It is known that mercury vapor greatly affects human health (Mantyla et al.,1976; Sexton et al.,1978; Nilsson et al.,1986; Piikivi et al.,1989; Osborne, 1992; Berglund et al., 1996; Stone et al., 2007). Even if taken orally, metal mercury is absorbed far less from the digestive tract and is less toxic than inorganic mercury or methyl mercury (Boening, 2000; Carrier G et al., 2001). On the other hand, mercury vapor is easily taken into the human body and has a long-term toxic effect thereon (Larsson, 1992; Clarkson, 1997; Morgan et al., 2006). However, sufficient data have not been obtained to date regarding the effects of mercury vapor on human development. We have tested the embryotoxicity of various dental materials by the embryonic stem cell test (EST) method, which is an in vitro embryotoxicity testing technique using the ES-D3 cells of embryonic stem cells derived from mice (Spielmann et al.,2001; Imai et al.,...

2001; Imai et al., 2006; Imai et al., 2007). However, the EST method cannot fully test the embryotoxicity of mercury vapor generated from dental materials such as dental amalgams. Therefore, with the aim of testing the *in vitro* embryotoxicity of mercury vapor, we tried to apply the rotary culture method, in which the culture bottle containing mercury vapor and culture medium was slowly turned and the cells come into direct contact with the gaseous phase containing mercury vapor.

**Materials and methods**

1. **Cell culture**

   The internal surface of a 1,950mL dry-sterilized rotary-culture bottle made of hard glass (New Brunswick, Canada), was thinly coated with type-I collagen gel (Cellmatrix, Nitta Geratin, Osaka) at freezing temperature. Into a culture medium containing Non-essential amino acid (NAA, Gibco, USA), β-mercaptoethanol (Gibco), L-glutamine-added DMEM (Gibco) and 20% FBS (HyClone, Canada) in volume ratio, 200 mL of ES-D3 cells (Fig. 1) adjusted to a concentration of $3.75 \times 10^4$ cells/mL were poured, and the bottle containing the above was sealed hermetically and subjected to culture at $37^\circ C$ for two hours in a rotary incubator (New Brunswick) turning at a rate of 4/7 revolutions per min.

2. **Exposure to mercury vapor**

   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 (Fig. 2). After sterilizing the entire unit with hot air, 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. Using a radiation thermometer (IT540S, Horiba, Kyoto), the temperature of the tip of the stainless-steel rod was confirmed to be above 480°C, that is, above 356.7°C, which is the evaporation temperature of mercury, and the evaporation of mercury was confirmed visually. After changing the lid, a rotary culture was done at $37^\circ C$ for three days in an incubator (New Brunswick). Three cul-

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Fig. 1  Embryonic stem cells, D3, obtained from Prof. Rolf Kemler (Max Planck Institute, Freiburg, Germany)

Fig. 2  Mercury evaporation equipment which improved the electronic heater of a soldering iron

Fig. 3  The rotational culture machine in $37^\circ C$ incubator
ture bottles were used for each of the two mercury exposure concentrations (Fig. 3). The cells inside the bottle were exfoliated using trypsin-added EDTA solution (Gibco), the solution for each culture bottle was poured into four 50mL centrifuge tubes, and each centrifuge tube was centrifuged at 800 revolutions per min for three minutes. The supernatant culture medium was exchanged with fresh culture medium.

3. Differentiation assay
A drop (20 mL) of the cell suspension was poured into the inside of the lid of a Petri dish 10 cm in diameter. After pouring 20 mL of PBS (-) into the Petri dish, the dish was covered with the lid to which the cells were attached and a hanging-drop culture was done in a carbon-dioxide incubator (Sanyo, Tokyo) for three days. The solution was poured into the centrifuge tube again, centrifuged, and exchanged with fresh culture medium. Five mL of the cell suspension were poured into an uncoated dish with a diameter of 6 cm for cell culture, and the suspension was cultured two more days. Six hundred ml of fresh culture medium were poured into all the wells of a 24well multiplate and the embryoid bodies (EBs) was placed into each well. After a static culture for five days, the existence of myocardial cell pulsation was examined in each cell under an inverted phase-contrast microscope (Olympus, Tokyo); that is, the ratio of the number of wells in which cell pulsation was observed to the number of wells in which EBs were successfully inoculated was examined. The control group was the group to which no mercury vapor was added.

4. Cell viability assay
Two hundred μL of cell suspension were poured into each well of a 96 well multiplate and a static culture was done for three days. After absorbing the entire culture medium in each well using a micropipette, 200 μL of new test solution were poured into each well and a static culture was done for two days. Five days after the culture, the culture medium was exchanged again with new test solution, and a static culture was done for five days. Twenty μL of a solution of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT, Nacalai tesque, Kyoto) in which MTT was dissolved into PBS at a rate of 5 mg/mL were added to each well via a micropipette, and the reaction was allowed to occur in an incubator for two hours. Then, the inside of each well was rinsed with PBS, the product was dissolved by adding 200 μL of dissolving solution (0.04 NHCl + isopropanol), and the absorbance at 570 nm was measured using a micro-plate reader (SPECTRA max PLUS, Molecular Devices, Canada) at a reference wavelength of 630 nm.

Results
1. Differentiation assay
As shown in Figure 4, in the groups where 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. Observation through the inverted phase-contrast microscope showed that, in some wells, EBs scarcely multiplied in the 5.0 μg group, whereas in the 2.0 μg group cell multiplication was observed in all the wells. However, during the test period, no pulsations were observed in the cell mass.

2. Cell viability assay
As shown in Figure 5, in the group where 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/m³, the cell viability was 96.0% of that of the control group.

Discussion
At the mercury exposure concentrations tested in this study, no cell pulsations were observed. The cell viability was affected to some extent in the 5.0 μg mercury concentration group, whereas in the 2.0 μg group little difference was observed from that of the control group. That is, while the mercury concentration affected the cell multiplication relatively slightly, it seriously affected the development of the ES cells. Even the mercury compounds examined by the conventional EST method showed strong embryotoxicity; the mercury concentrations of both IC50 and ID50 were 0.2-0.25 μg (Imai et al., 2006) . Since the cells were directly exposed to mercury vapor in this study and the exposure time differed between this study and past studies, a direct comparison with past test results is inappropriate; however, regarding the impairment of the expression of pulsation in differentiated myocardial cells, a strong effect was observed.

The level at which the inhalation of mercury vapor becomes toxic to humans is 1.2-8.5 mgHg/m³, and the level at which the inhalation of mercury vapor has no effect on humans is 0.1 mg/m³. Mercury evaporates easily, and its saturation concentration at room temperature is 20 mg/m³, which is 400 times the allowable concentration (0.05 mg/m³) in the working environment (http://www.umin.ac.jp/chudoku/chudokuinfo/q/q121.txt). Mercury is used in various products such as fluorescent lights or lamps, mercury lamps, old-style thermometers, stamp pads and barometers. Above all, fluorescent lights are widely used, and when they are lit, a considerable amount of mercury vapor is present inside the lamps.

For the past 150 years, dental amalgam has been widely used as a filling material for treating caries, etc (Phillips et al. 1949, Smith, 1967). Dental amalgam is made by kneading mercury with an alloy of silver, tin and copper. However, since dental amalgam contains a great deal of mercury, there have been numerous reports of its toxicity. Recently, in Japan, dental materials such as composite resins and glassionomer cements have begun to be widely used, and dental amalgam is now rarely chosen as a restoration material. However, when previously existing dental amalgam is removed using an air turbine and running water, it is highly probable that the wastewater from the dental clinic will contain mercury, so there is a danger of environmental pollution. Also, dentists and dental staff may be affected by the inhalation of dust and mercury vapor produced at the time of removal. Mercury vapour concentrations in air concentrations were measured at the breathing zone of the dentist during continuous operation of the aspirator. At the dentist's breathing zone, mercury vapour concentrations of ten times the current occupational exposure limit of 2.5 μg/m³ were recorded after 20 minutes of continuous aspirator operation (Stonehouse et al., 2001).

Mercury vapor released from a single amalgam restoration in pregnant rats and mercury concentrations in maternal and fetal rat tissues were studied (Takahashi et al., 2001). They reported the highest mercury concentration among fetal organs was found in the liver, the kidneys and brain. Furthermore, since there is a possibility that mercury vapor is generated from the dental amalgam in the body when the remains are cremated, the staff working at crematories may be endangered and the environment around crematories may be polluted.

Although mercury vapor was observed to strongly impair the differentiation of EBs in this study, the test results this time did not prove that mercury vapor has a strong embryotoxic effect on humans. Regarding the testing method, the test used in this study was quite different from other tests, in that cells were directly exposed to mercury vapor. We think it important to develop a device that closely mimics the actual exposure conditions of the human body, including an exposure method that is different from that used in this study, as an alternative to animal experiments.

Acknowledgments
The authors would like to thank Dr. Horst Spielmann, Honorary Professor of the Free University of Berlin, Germany.

The present study was partly supported by the Japanese Society of Alternatives to Animal Experiments, Grant for Child Health and Development (19C-4) from the Ministry of Health, Labour and Welfare in Japan, Grant-in-Aid for General Scientific Research (No.19592272) from the Ministry of Education, Science, Sports and Culture in Japan, and "High-Tech Research Center" Project for Private Universities: matching found subsidy from MEXT, 2007-2011. This study was carried out using the Institute of Dental Research, Osaka Dental University.
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(Received: September 6, 2008/ Accepted: December 23, 2008)

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