The plasticizer di(2-ethylhexyl)phthalate (DEHP) used in peritoneal dialysate bags is known to dissolve into the solution, even though the quantity is small. Long-term exposure of the peritoneum to DEHP could be a cause of peritoneal deterioration. Although some papers have been published about DEHP toxicity to the peritoneum, acute DEHP toxicity is not fully understood to date. We therefore conducted the present in vitro study, using peritoneal mesothelial cells to examine acute DEHP toxicity.

Peritoneal mesothelial cells were harvested from the peritonea of Sprague–Dawley rats and were cultured in Dulbecco modified eagle medium supplemented with 1% fetal calf serum. Confluent mesothelial cells were incubated with DEHP at various concentrations for 24 hours. The concentration of lactate dehydrogenase in the supernatant was measured and compared to control samples. In addition, apoptosis of mesothelial cells was examined by the TUNEL method at various concentrations of DEHP.

We found that DEHP (260 pmol/L to 2.6 mmol/L) neither elevated lactate dehydrogenase in the supernatant nor induced apoptosis of cultured mesothelial cells. The concentration of DEHP in peritoneal dialysate bags is reported to be approximately 20 nmol/L (Terumo Corporation, Tokyo, Japan, personal communication), long-term exposure to DEHP may have a harmful effect on peritoneal cells. A previous in vitro study reported that DEHP induced peritoneal damage (2), and another clinical study implicated DEHP eluted from the hemodialysis blood circuit in the promotion of atherosclerosis in hemodialysis patients (3). In the present study, we evaluated DEPH toxicity through in vitro experiments on rat peritoneal mesothelium.

Materials and methods

Preparation of rat peritoneal mesothelial cells
Male 8-week-old Sprague–Dawley rats were obtained from Charles River Japan (Yokohama, Japan). Peritoneum removed from ether-anesthetized rats was treated with 0.5 mg/mL collagenase (Sigma–Aldrich, Tokyo, Japan), and the mesothelial cells were harvested. Cells were maintained in Dulbecco modified eagle medium (DMEM: Invitrogen, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS: Invitrogen) and bubbled with 5% CO₂ at 37°C. All studies described here were performed on cells between the 4th and 7th passages.

DEHP toxicity test
We obtained DEHP from Wako Pure Chemical Industries (Osaka, Japan). The DEHP was dissolved in ethanol (EtOH) at 50% volume. After a 24-hour incubation in DMEM and 10% FCS in a 96-well
plate, cells were washed with DMEM and 1% FCS once, and incubated in 100 µL DMEM and 1% FCS, with DEHP added at various concentration. The same incubation study was also performed with PD solutions that are clinically available worldwide.

After incubation in the experimental conditions, the lactate dehydrogenase (LDH) concentration in the supernatant was determined using a Cytotoxicity Detection Kit PLUS (Roche Diagnostics, Tokyo, Japan). Briefly, the culture media were collected and centrifuged at 1500 rpm for 5 minutes to pellet the cell debris. Assay solution for LDH (80 µL) was added to the isovolume supernatant and was incubated at 37°C for 30 minutes. Absorbance was measured at 492 nm and 620 nm, and the LDH level was calculated in reference to a standard assay. Cytotoxicity was calculated as follows:

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\text{cytotoxicity (\%) = \frac{(\text{experiment} - \text{negative control})}{(\text{positive control} - \text{negative control})} \times 100}
\]

The positive control refers to cells incubated with 1% TritonX-100–supplemented DMEM and 1% FCS for 2 hours. The negative control refers to cells incubated with only DMEM and 1% FCS.

**Apoptosis**

After the experimental incubation, cells were centrifuged at 1500 rpm for 5 minutes, and the culture media were removed. Cells were frozen at –80°C for 2 hours, and cell apoptosis was determined by the TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling kit: Wako Pure Chemical Industries).

**Results**

**Preliminary experiments**

First, we evaluated the cytotoxicity of the EtOH solvent. Rat mesothelial cells grown in a 96-well microplate were incubated for 6 hours in DMEM and 1% FCS with added EtOH at increasing concentrations. The LDH concentration in supernatant was then measured. The EtOH was cytotoxic to mesothelial cells at concentrations of 5% or more (Figure 1).

To exclude the influence of EtOH on cells, the final EtOH concentration in all experimental solutions

![Cytotoxicity effect of ethanol (EtOH) on rat peritoneal mesothelial cells. Rat mesothelial cells grown on 96-well microplates were incubated for 6 hours in Dulbecco modified eagle medium supplemented with 1% fetal calf serum to which was added varying concentrations of EtOH. Lactate dehydrogenase levels in supernatants were then determined using an analysis kit. At concentrations of 5% or more, EtOH was cytotoxic to the rat peritoneal mesothelial cells. To exclude the influence of EtOH on cells, the final EtOH concentration was adjusted to less than 2.5% in all experiments described in the present paper.](image)
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Evaluation here was adjusted to below 2.5%. And because DEHP becomes an emulsion in culture medium, a concentration of 86 mmol/L or more could influence the visible wavelength absorbance during LDH measurement [Figure 2(a)]. Therefore, given that DEHP is dissolved into EtOH volume for volume, the final concentration of the dissolved DEHP was adjusted to 65 mmol/L with 2.5% EtOH.

Next, we added the DEHP–EtOH to the 1% TritonX-100–treated positive control cells and examined the influence of DEHP on the LDH assay. The assay was found to be negatively affected at concentrations of DEHP above 8.6 mmol/L [Figure 2(b)].

Based on these preliminary experiments (Figures 1 and 2), cytotoxicity tests were performed at DEHP concentrations below 2.6 mmol/L.

The final preliminary experiments were used to determine the incubation period and cell density for the DEHP cytotoxicity assay. No LDH assay differed substantially for cell confluences between 70% and 100%. Similarly, incubation time (6, 24, and 48 hours) did not influence the results of LDH assays on mesothelial cells treated with DEHP (Figure 3). Incubation studies were therefore performed at conditions of 100% confluence and 24-hours of incubation with DEHP–EtOH.

**Cytotoxicity of DEHP**

Rat peritoneal mesothelial cells were incubated with DEHP at various concentrations for 24 hours. Figure 4 shows that DEHP at levels ranging from 260 pmol/L to 2.6 mmol/L does not have a cytotoxic effect on rat peritoneal mesothelial cells.

The same assays were used to determine the cytotoxic effect of clinically available PD fluids (Figure 4, right-hand panels). One solution was pH-neutral (6.3–7.3); the other was a conventional acid solution. The neutral solution had a more favorable effect on cytotoxicity after a 1-hour incubation, but no difference in cytotoxicity was apparent after a 24-hour incubation.

**DEHP-induced apoptosis**

The DEHP-induced apoptosis was determined by the TUNEL method (Figure 5), and DEHP in the range

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**FIGURE 2** Optical density (OD) interference by di(2-ethylhexyl)phthalate (DEHP) dissolved in ethanol (EtOH) on lactate dehydrogenase (LDH) measurement. (A) Concentrations of DEHP of 86 mmol/L (mM) influenced the results of LDH measurement. At concentrations greater than millimoles, DEHP cannot be dissolved in culture media; instead, it forms an emulsion, which may be the reason that it interferes with LDH determination. (B) Varying concentrations of DEHP–EtOH were added to 1% TritonX-100–treated positive control cells, and concentrations above 8.6 mM were observed to influence the results of LDH measurement. To exclude the influence of DEHP on LDH measurement, the final DEHP concentration was adjusted to less than 2.6 mM. * p < 0.05.
of concentrations tested did not increase apoptosis in the rat peritoneal mesothelial cells.

Figure 5 also shows the time course of mesothelial apoptosis induced by PD solution. Peritoneal dialysate does not induce apoptosis of mesothelial cells for up to 24 hours.

**Discussion**

The plasticizer DEHP is one of the constituents of polyvinyl chloride peritoneal dialysate bags. Because previous reports warned that DEHP might have a harmful effect on peritoneal cells, we set out to determine the cytotoxicity of DEHP in an *in vitro* study. In some cells, DEHP induces apoptosis, but it suppresses apoptosis in other cells (4–7).

The present study revealed that DEHP up to 2.6 mmol/L does not have a cytotoxic effect and does not induce apoptosis in rat peritoneal mesothelial cells. Although the TUNEL method can misidentify necrosis as apoptosis (false positive), DEHP did not influence apoptosis at all in the mesothelial cells used in this study (8,9).

Because the concentration of the DEHP dissolved from a peritoneal dialysate bag into the PD solution is reported to be very low (approximately 20 nmol/L), exposure to DEHP for up to 48 hours in the course of PD is unlikely to have a harmful effect on peritoneal mesothelial cells. But the long-term effects of DEHP must be determined in future experiments.

In addition to mesothelial cells, monocytes—in particular, macrophages—are known to play an early and major role in the host defense to foreign bodies and to many stimuli from outside the peritoneum. Macrophages use phagocytosis to combat foreign bodies directly and may secrete cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor in response to immunologic stimuli. Plasticizers are known to stimulate secretion of cytokines by monocytes. Fracasso *et al.* (2) reported that DEHP at 2.8 mmol/L increased IL-1 secretion from mononuclear cells. But given that DEHP cannot be fully dissolved in water–EtOH and is present as an emulsion at 2.6 mM, the speculation is that monocytes may be reacting to the DEHP emulsion as a foreign body.

**Conclusions**

The present study indicates that DEHP dissolved in peritoneal dialysate used clinically does not present an acute cytotoxic risk to the peritoneal mesothelium.
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FIGURE 4  Cytotoxic effect of di(2-ethylhexyl)phthalate (DEHP) on rat peritoneal mesothelial cells. Rat mesothelial cells grown on 96-well microplates were incubated for 24 hours in Dulbecco modified eagle medium supplemented with 1% fetal calf serum to which was added varying concentrations of DEHP. Lactate dehydrogenase levels in supernatants were then determined using an analysis kit. At concentrations of 260 pmol/L (pM) to 2.6 mmol/L (mM), DEHP had no cytotoxic effect on rat peritoneal mesothelial cells. “Control” is a positive control treated with 1% TritonX-100. The cytotoxic effects of commercial peritoneal dialysis (PD) solutions (one neutral, one acidic) were also evaluated. In a 1-hour incubation, neutral solution had a more favorable effect than acidic solution did; in a 24-hour incubation, no difference in cytotoxicity between the neutral and acidic solutions was observed. All values are mean ± standard error of the mean. * Nonsignificant; ** p < 0.05. OD = optical density.

FIGURE 5  Apoptosis in rat peritoneal mesothelial cells in the presence of di(2-ethylhexyl)phthalate (DEHP). Rat mesothelial cells grown on 96-well microplates were incubated for 24 hours in Dulbecco modified eagle medium supplemented with 1% fetal calf serum to which was added varying concentrations of DEHP. After a period of freezing at –80°C, cell apoptosis was determined using a TUNEL method kit. At concentrations of 260 pmol/L (pM) to 2.6 mmol/L (mM), DEHP did not increase the level of apoptotic cells observed. Levels of cell apoptosis induced by commercial peritoneal dialysis (PD) solutions (one neutral, one acidic) were also evaluated. In 1-hour and 24-hour incubations, neither solution induced significant apoptosis. All values are mean ± standard error of the mean. * Nonsignificant. OD = optical density.
References
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