

Original Article

Cytotoxic Effects of Alcohol Extracts from a Plastic Wrap (Polyvinylidene Chloride) on Human Cultured Liver Cells and Mouse Primary Cultured Liver Cells

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An increasing accumulation of microplastics and further degraded nanoplastics in our environment is suspected to have harmful effects on humans and animals. To clarify this problem, we tested the cytotoxicity of two types of plastic wrap on human cultured liver cells and mouse primary cultured liver cells. Alcohol extracts from plastic wrap, *i.e.*, polyvinylidene chloride (PVDC), showed cytotoxic effects on the cells. Alcohol extracts of polyethylene (PE) wrap were not toxic. The commercially available PVDC wrap consists of vinylidene chloride, epoxidized soybean oil, epoxidized linseed oil as a stiffener and stabilizer; we sought to identify which component(s) are toxic. The epoxidized soybean oil and epoxidized linseed oil exerted strong cytotoxicity, but the plastic raw material itself, vinylidene chloride, did not. Our findings indicate that plastic wraps should be used with caution in order to prevent health risks.

Key words: plastic wrap, plasticizer, cytotoxicity, liver cells, in vitro

Approximately 448 million tons of plastic were produced worldwide in 2015, and about half of that was disposed of in the environment. It is not known how long it will take for the plastic that has been disposed of to completely biodegrade into its constituent molecules, and the disposal of plastic waste has thus become an important environmental and public health issue [1]. When plastics that have been disposed of are broken down into microplastics and further into nano-sized substances by natural forces such as sunlight, waves, wind, and heat, some biological effects are expected.

In 2016, microplastics were found in the digestive tracts of Japanese anchovies sampled from Tokyo Bay [2], and many other cases of microplastic ingestion by fish are cited in that report. Jeong *et al.* reported that marine copepods that engulfed microplastics exhibited reduced growth and fecundity, possibly due to a physical disturbance of the digestive system. They also demonstrated that microplastics could enhance the functions of oxidative stress-induced MAPK/Nrf2 pathway-mediated genes, but the relationships between the altered genetic functions and subsequent biological effects such as reductions of growth rate and fecundity are not clear [3]. Lithner *et al.* observed the acute tox-

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icity of leachates from many plastic products by evaluating the immobility of the small crustacean *Daphnia magna* [4].

Plastics on the market usually contain plasticizers such as dibutyl phthalate, diethylhexyl phthalate, butyl benzyl phthalate, and bisphenol A. Oehlmann *et al.* investigated the biological effects of plasticizers on wildlife, including molluscs, crustaceans, insects, fish, and amphibians. Some of the organisms they examined showed reduced reproduction and genetic aberrations, and most of the plasticizers appeared to interfere with the functioning of various hormone systems [5].

Although there are a few reports on the harmful effects of microplastics on some organisms, no cell-level studies have been reported. Herein we investigated whether two types of commercially available plastic wrap, *i.e.*, polyvinylidene chloride (PVDC) and polyethylene (PE), have any effects on human and mouse liver cells in culture. We selected these cell types because the liver plays a central role in drug metabolisms. Ideally, normal human liver cells would be the most suitable for toxicological studies, but such cell lines are not available at this time. We thus used the human hepatoma cell line HLE [6].

Materials and Methods

Cells and culture. To test the cytotoxic effects of the components of plastics, we used the human hepatoma cell line HLE and mouse primary cultured liver cells. The HLE cells were cultured in Minimum Essential Medium Eagle (MEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in 5% CO₂.

The experimental protocols were approved by the Animal Care and Use Committee of Okayama University (approval no. OKU-2017250). Mice were treated painlessly or with anesthesia according to the strict guidelines of the Policy on the Care and Use of the Laboratory Animals of Okayama University. For the culture of mouse primary liver cells, the liver of a C57BL/6J mouse (7 weeks old; CLEA Japan, Tokyo) was minced and treated with collagenase (1 mg/ml; Wako, Hiroshima, Japan) in D/F medium (Thermo Fisher Scientific, Waltham, MA) at 4°C for 24 h. After the digested solution was passed through a 70- μ m filter (Corning, Corning, NY), the collected cells were cultured using D/F medium with 10% FBS.

Preparation of assay samples. Two types of wrap were examined: PE, which consists of ethylene and fatty acid ester as a softener, and PVDC, which is composed of vinylidene chloride, epoxidized soybean oil, and epoxidized linseed oil as a stiffener and stabilizer. PE and PVDC plastic wraps were obtained from Pax-Asian (Hiroshima, Japan) and KUREHA (Tokyo), respectively. Epoxidized soybean oil and epoxidized linseed oil were obtained from Adeka (Saitama, Japan).

One gram of wrap that had been finely cut with scissors was immersed in 10 ml of a solution (hot water, microwaved hot water, phosphate-buffered saline [PBS], vinegar, and methanol) for 24 h. After centrifugation, each sample was used for the examination illustrated in Fig. 1. PBS sample was adjusted to pH 1.0 by HCl (11.2 N; Wako) (Fig. 1; described as PBS pH 1.0). Methanol was used as a substitute for oils and fats. Compared to ethanol, methanol is less toxic to cultured cells, because the cultured cells have no enzymes to metabolize methanol to toxic substances such as formaldehyde and formic acid.

In the case of water, 1 g of the cut wrap was boiled or microwaved (90 sec) and then kept at room temperature for 24 h. For the experiments other than the one shown in Fig. 1, PE and PVDC samples were centrifuged and the collected supernatant was evaporated. The remaining residue was dissolved in 1 ml of PBS and used as a test sample (Figs. 2 and 3). These experiments were carried out with a plastic (polypropylene) test tube. No cytotoxic effects were detected from methanol alone put in the test tube for 24 h.

One milliliter each of vinylidene chloride (1,1-dichloroethylene; Sigma-Aldrich), epoxidized bean oil, and epoxidized linseed oil was dissolved in 10 ml of PBS. The solutions were then vigorously shaken and used as test samples at the concentrations indicated in the Results section.

Cytotoxicity assay. For the evaluation of cytotoxicity, 500 cells were seeded into 60 mm plastic dishes with 4 ml of culture medium and placed in a CO₂ incubator. At 24 h later, the test samples dissolved with PBS were added to cultures. After 1 week, the colonies were stained with 1% crystal violet (Sigma-Aldrich) solution, and the number of colonies was counted under a light microscope. The colony number of the control cultures was taken as 100%, and the colony numbers of the assay samples were compared with the control. Morphological changes were observed by light

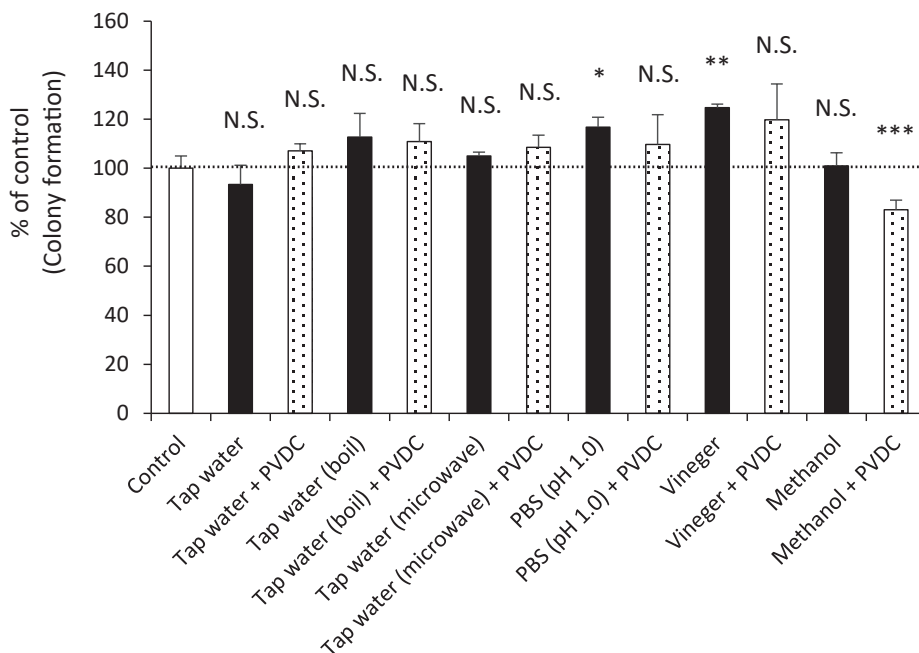


Fig. 1 Effects of various solvent extracts of polyvinylidene chloride (PVDC) on human cultured liver cells. Five hundred cells/60 mm dish were seeded with 4 ml of culture medium; 24 h later the cells were treated with 200 μ l with/without each PVDC extract for 7 days. The cells were fixed with 100% ethanol and stained by 1% crystal violet. Data are the mean \pm SD of three separate experiments. N.S.: Not significant, * p <0.05, ** p <0.01, *** p <0.001.

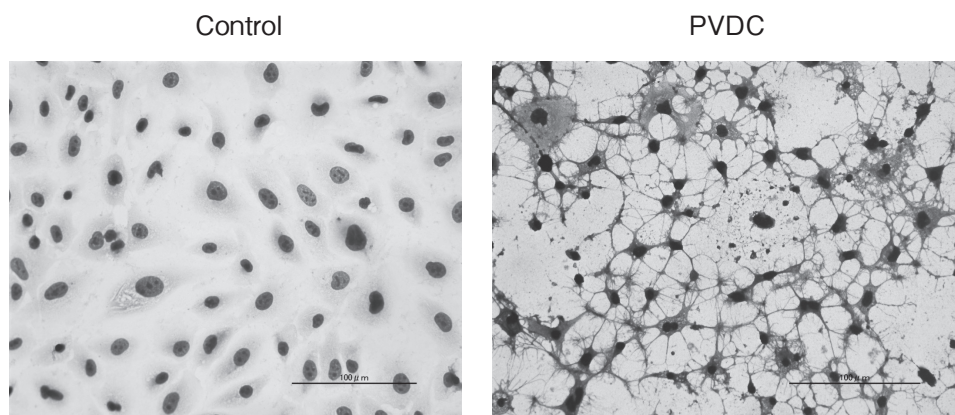


Fig. 2 The degenerative cells treated for 3 days with alcohol extracts of PVDC. HLE cells grown to confluence in a dish with 4 ml of culture medium were treated with 200 μ l of an alcohol extracts (described in Materials and Methods) of PVDC. After 72 h, the cells were fixed with 100% ethanol and stained with 2% Giemsa solution. Scale bar: 100 μ m.

microscopy. The cells were fixed with 100% ethanol and stained with 2% Giemsa (Merck, Darmstadt, Germany) solution.

Apoptosis assay. First, Hoechst staining was done to detect apoptotic cells. HLE cells and mouse primary liver cells were treated with methanol, PE and PVDC extracts for 72 h. The cells were then incubated in Hoechst33342 solution (Thermo Fisher Scientific) at

a final concentration of 5 μ g/ml for 30 min. Apoptotic cells were observed by fluorescence microscopy and evaluated as a percentage of total cells.

We then performed a western blot analysis to observe the apoptotic signals of HLE and mouse primary liver cells treated with the PE and PVDC extracts. The antibodies used were as follows (all from Cell Signaling Technology, Beverly, MA unless otherwise

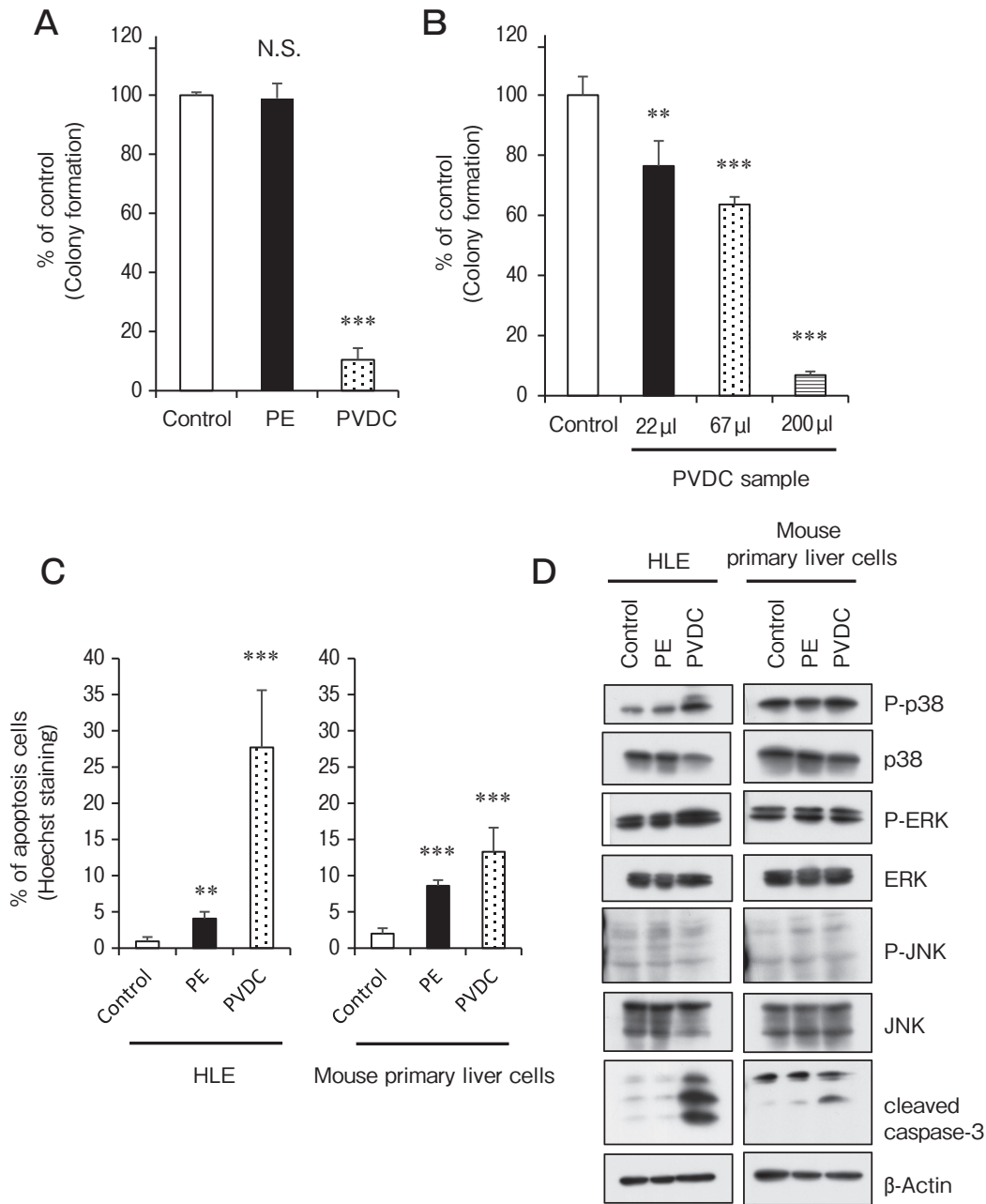


Fig. 3 Cytotoxic effects of methanol extracts of PVDC on human cultured liver cells. **A**, Five hundred cells/60 mm dish were seeded with 4 ml of culture medium; 24 h later the cells were treated with 200 µl of each concentrated sample for 7 days. The cells were fixed with 100% ethanol and stained by 1% crystal violet. Data are the mean \pm SD of three separate experiments. *** p <0.001; **B**, Five hundred cells/60 mm dish were seeded with 4 ml of culture medium and treated 24 h later with 200 µl (original), 67 µl (1/3), or 22 µl (1/9) of the test sample for 7 days. In the case of the 67 µl and 22 µl treatments, 133 µl and 178 µl of PBS was added, respectively. The cells fixed with 100% ethanol were stained by 1% crystal violet. Data are the mean \pm SD of three separate experiments; ** p <0.01, *** p <0.001; **C**, Fifty thousand cells/well (6-well plate) were seeded with 2 ml of culture medium and then treated 24 h later with 100 µl of each concentrated sample for 3 days. Apoptotic cells stained with Hoechst33342 (5 µg/ml) were counted. Data are the mean \pm SD from three separate images. ** p <0.01, *** p <0.001; **D**, Fifty thousand cells/well (6-well plate) were seeded with 2 ml of culture medium and treated 24 h later with 100 µl of each concentrated sample for 24 h. The expressions of p38 MAPK, SAPK/JNK, p44/42 MAPK (Erk1/2), cleaved caspase-3, and β -Actin were analyzed by western blotting.

noted): rabbit anti-cleaved type caspase-3 antibody, rabbit anti-SAPK/JNK antibody, rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody, rabbit anti-p38 MAPK antibody, rabbit anti-phospho-p38 MAPK (Tyr182) antibody, rabbit anti-p44/42 MAPK (Erk1/2) antibody, rabbit anti-phospho-p44/42 MAPK (T202/Y204) antibody, and mouse anti-beta-actin antibody (Sigma-Aldrich).

Mass analyses by MALDI-TOF-MS. Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) (AXIMA Performance, Shimadzu, Kyoto, Japan) was performed to verify the molecular mass of the epoxidized oils and plastic extracts. We used 2,5-dihydroxybenzoic acid (DHB) (Shimadzu) as the matrix, and 0.5% (v/v) formic acid was used as the doping agent. Sample mixtures were prepared by mixing matrix: sample: 0.5% formic acid at a ratio of 25 : 5 : 1, and 1 μ l of each sample mixture was loaded onto the MALDI-TOF-MS sample plate. A Positive ion source in linear mode and power in the range of 120-125 mV were used. Each spot was analyzed using a random raster of 200 profiles, and each profile consisted of data from five laser shots. The mass-to-charge ratio (m/z) was obtained through external calibration, by a DHB matrix.

Ames test. *Salmonella enterica subspecies I, serovar Typhimurium* (*Salmonella typhimurium*) strains TA98 [*hisD3052 Δ uvrB gal bio chl1005 rfa1001/pKM101*] and TA100 [*hisG46 Δ uvrB gal bio chl1005 rfa1001/pKM101*] were gifts from Dr. B.N. Ames of the University of California, Berkeley [7]. *S. typhimurium* YG7108 [*hisG46 Δ uvrB gal bio chl1005 rfa1001 Dada_{st}:: Km^r Dogt_{st}:: Cm^r*] was a gift from Dr. M. Yamada (National Institute of Hygienic Sciences, Tokyo) [8]. The supernatant fraction of rat liver homogenate (S9) was prepared from Sprague-Dawley rats (6-week-old males) that had been induced by phenobarbital and 5,6-benzoflavone. Positive controls, *i.e.*, 2-aminoanthracene (2AA), methylnitrosourea (MNU), 2-nitrofluorene (2NF), and N-nitrosomorpholine (NMOR), were purchased from commercial sources.

The Procedures for the genotoxicity assays were based on the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) harmonized tripartite guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2 (R1) Current Step 4 version dated 9 November 2011 [Last access: 17

March 2020, <http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html>]. The recommended set of bacterial strains includes those that detect base substitution and frameshift mutations with and without metabolic activation, and appropriate positive and negative controls. We selected the strain *S. typhimurium* TA98 for the detection of frameshift mutations and *S. typhimurium* TA100 for the detection of base substitution mutations. We used *S. typhimurium* YG7108, a highly sensitive strain, for the detection of mutagenic alkylating agents. All assays were performed with plate-incorporation methods [7] with and without metabolic activation (hereafter referred as +S9 and -S9, respectively). The assays were performed in duplicate.

Statistical analysis. Data are represented as the mean \pm SD of three separate experiments. All data were analyzed by unpaired Student's *t*-test for significant differences between the mean values of each group.

Results

Many types of food are packaged and sold with plastic wrap, and we hypothesized that some cytotoxic substances might migrate from the plastic wrap to the food. Herein, we examined the effects of PVDC extracts in various solvents (tap water, boiled tap water, microwaved water, PBS, vinegar, methanol) on human liver cells in culture. As shown in Fig. 1, the methanol extracts of PVDC caused a significant decrease in colony formation, whereas the other extracts did not. The cells treated with the PVDC sample morphologically showed degenerative changes, and most of the cells appeared to be dying (Fig. 2).

As shown in Fig. 3A, the methanol extracts of PE showed no cytotoxic effects on the cultured liver cells (the HLE line), but the methanol extracts of PVDC caused serious damage to the colony formation. This cytotoxicity of the PVDC extracts was dose-dependent (Fig. 3B).

To learn how the cells die due to PVDC alcohol extracts, we conducted an apoptosis assay of HLE and mouse primary liver cells by Hoechst staining. As shown in Fig. 3C, the PVDC extracts induced apoptosis in both HLE and mouse primary liver cells. The rates of apoptotic cells in the HLE and mouse primary liver cells were approx. 30% and 15%, respectively. Unexpectedly, the PE extracts induced apoptosis of these cells to a certain degree, though they did not significantly decrease

the colony formation.

We next performed a western blot analysis to confirm cell death signaling. As shown in Fig. 3D, when the HLE and mouse primary liver cells were treated with PVDC extracts, they showed caspase-3 activation. We also observed that p38 MAPK phosphorylation, upstream of caspase-3, was activated only in HLE cells treated with PVDC extracts, and not in mouse primary liver cells. These findings indicated that the PVDC extracts induced apoptosis to some degree. Regarding other signals, no significant changes were observed.

Since PVDC wrap consists of vinylidene chloride, epoxidized soybean oil, and epoxidized linseed oil as a stiffener and stabilizer, we studied the cytotoxicity of each of these components, and the results demonstrated that the epoxidized soybean oil and epoxidized linseed oil showed cytotoxicity, but vinylidene chloride itself did not (Fig. 4).

These findings led us to perform MALDI-TOF-MS to determine whether the epoxidized oils were present in the PVDC alcohol extracts. The results of the molecular mass analysis using these plasticizer and alcohol extract samples are shown in Fig. 5. Some spectrum peaks of epoxidized soybean oil were observed in the PVDC alcohol sample's spectrum (1056 m/z and 1088 m/z) but not in the PE alcohol sample's spectrum.

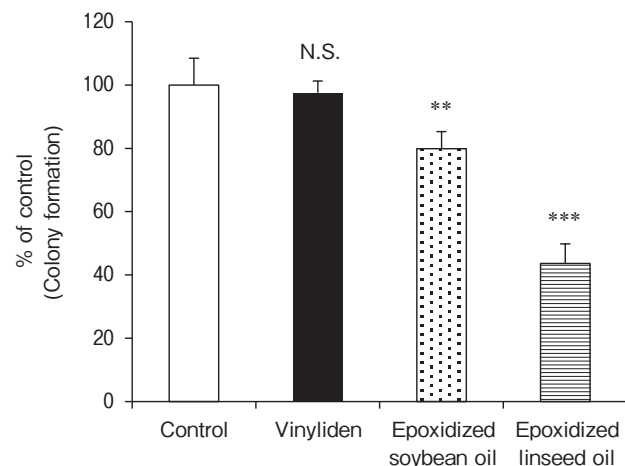


Fig. 4 Effects of each ingredient of PVDC wrap on human cultured liver cells. Five hundred cells/60 mm dish were seeded with 4 ml of culture medium and treated 24 h later with PBS, polyvinylidene chloride, epoxidized soybean oil, and epoxidized linseed oil at a final concentration of 0.25% for 7 days. The cells fixed with 100% ethanol were stained by 1% crystal violet. Data are the mean \pm SD of three separate experiments. ** $p < 0.01$, *** $p < 0.001$.

but no spectrum peak was matched to the PE or PVDC alcohol extract sample's spectrum (data not shown). These results showed that epoxidized soybean oil was present in the PVDC alcohol extract samples.

The results of our evaluation of the genetic effects of the alcohol extracts are shown in Table 1: no increase in bacterial mutation was observed with *S. typhimurium* TA98 nor *S. typhimurium* TA100 with or without metabolic activation compared to those with solvent controls.

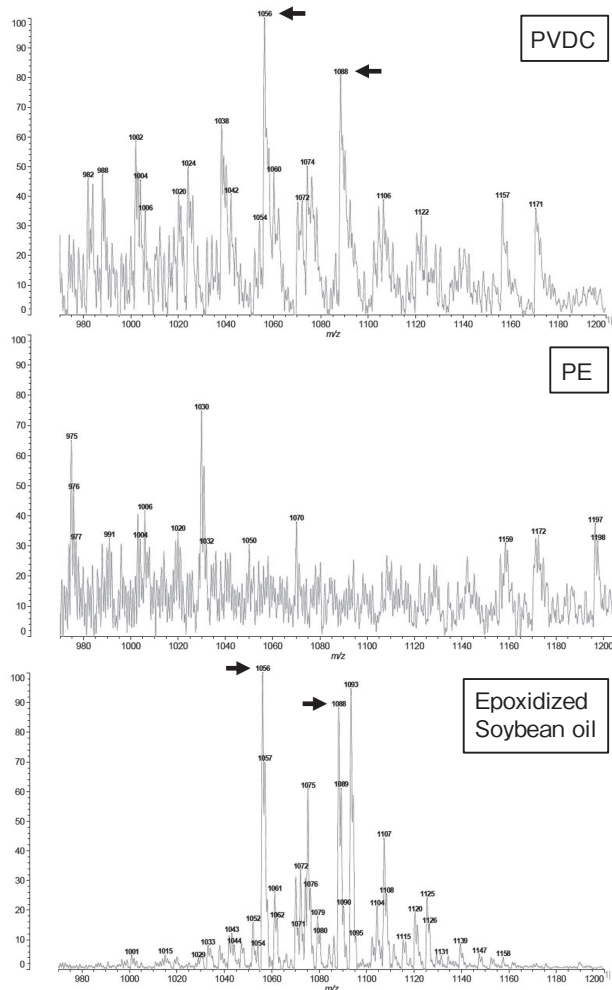


Fig. 5 Analysis of alcohol extracts of PE and PVDC by MALDI-TOF-MS. Epoxidized soybean oil has an estimated molecular weight of 988 g/mol. Under positive ionization mode, several cationic adducts of epoxidized soybean oil were postulated: 1056 m/z $[M-H+3Na]^+$, 1075 m/z $[M+K+2Na+2H]^+$, 1088 m/z $[M-H+2K+Na]^+$, and 1093 m/z $[M+2K+Na+4H]^+$. Cationic adducts of epoxidized soybean oil, at 1056 m/z $[M-H+3Na]^+$ and 1088 m/z $[M-H+2K+Na]^+$, were observed in PVDC plastic extracts as indicated by arrows.

Table 1 Genotoxicity of alcohol extracts of polyvinylidene chloride in the Ames test

Test sample, ml	TA98		TA100		YG7108	
	+S9 ^a	−S9 ^b	+S9	−S9	+S9	−S9
No. of revertants/plate						
0.1	39, 39	14, 11	141, 158	93, 102	10, 13	9, 8
0.01	21, 40	14, 13	146, 184	118, 126	6, 6	10, 10
0.001	26, 44	12, 16	156, 153	112, 135	8, 9	1, 10
Solvent control:						
H ₂ O, 0.1 ml	37, 32	6, 35	140, 176	97, 111	20, 11	7, 6
Positive control:						
2AA, 100 μM 0.1 ml	642, 638	nd ^c	489, 918	nd	nd	nd
2NF, 50 μM 0.1 ml	nd	253, 211	nd	269, 281	nd	nd
NMOR, 100 mM 0.1 ml	nd	nd	nd	nd	875, 1,334	nd
MNU, 100 mM 0.1 ml	nd	nd	nd	nd	nd	527, 459

^aThe method of preparation is described in the Materials and Methods.

^b+S9: with metabolic activation.

^c−S9: without metabolic activation.

nd: not done.

Discussion

Harmful substances associated with plastics can be divided into three categories: (1) ingredients of the plastic material, (2) byproducts of manufacturing, and (3) chemicals adsorbed from the environment. Our present findings demonstrated that alcohol extracts of commercially available PVDC wrap exerted harmful effects on the cultured human liver cells and mouse liver cells, and that the cytotoxic effects were due to the additives to PVDC, *i.e.*, epoxidized soybean oil and epoxidized linseed oil as a stiffener and stabilizer.

Although our experimental conditions (*e.g.*, treating the plastic wrap with alcohol) may not occur in daily life, plastics that have been disposed of eventually completely degrade into their constituent ingredients, and thus some substances that are harmful to organisms may be released into the environment. It should be determined whether or not these finely degraded materials are toxic to cells, but it is very difficult to collect these precise ingredients from the environment. This is why we examined the cytotoxicity of various solvent extracts of plastic wrap. Some substances that are hazardous to human cells might be transferred from plastic wrap to the food it surrounds.

We observed that the cells treated with alcohol extracts of PVDC morphologically appeared degenerative. Considering that the cytotoxic additives epoxi-

dized soybean oil and epoxidized linseed oil both contain fatty acids, they can integrate into the cell membrane, resulting in a disturbance of membrane functions. Thus, degenerative changes of the cells may be due to membrane damage, indicating that most of the dying cells were probably necrotic, though some were apoptotic. Thus, when a fatty food is wrapped with this type of wrap, some toxic substances might move from the wrap into the food.

We speculated that the cytotoxic substances of the PVDC alcohol extract could induce genetic effects and therefore conducted Ames tests, but we observed no mutagenic effects. Coincidentally, no relationship between the occurrence of cancer in humans and occupational exposure to vinylidene chloride has been demonstrated (IRIS Chemical Assessment summary on 1,1-Dichloroethylene, U.S. Environmental Protection Agency National Cancer for Environmental Assessment). However, mesothelioma in rats treated with vinylidene chloride and renal carcinomas in mice treated with the same material have been reported [9,10]. Since the present experiment for detecting toxicity with cultured cells and genetic effects by the Ames test is a short-term assay system, it is not suitable for determining the carcinogenicity of test materials because animal studies on carcinogenesis require a long time to obtain results. Thus, the cancer risks of plastic products for humans should be examined in future studies.

Three methods of reducing plastic hazards have been proposed. The first is to use less plastics. The second is to develop a closed-loop system in which all plastics are reused and recycled. However, such a system would involve the use of a great deal of energy, resulting in greenhouse gas emissions. The third method is to produce biodegradable plastics. However, biodegradation also takes time, and if biodegradable plastics are mixed with conventional plastics in the recycling process, the process would not work well. Therefore, the reduction of the use of plastics in our lives is the most achievable goal.

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