

Cytocompatibility of New Phthalate Ester-free Tissue Conditioners *in vitro*

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The estrogenic activity of phthalate esters for dental use as plasticizers was concerned. To study the feasibility of candidate chemicals for phthalate tissue conditioners, three candidate plasticizers, di-n-butyl sebacate, acetyl tributyl citrate and tri-n-butyl phosphate together with conventional plasticizers were examined for estrogenic activity and cytotoxicity using three estrogenicity assays, human gingival fibroblasts and living skin equivalent. None of the three candidate plasticizers showed any estrogenic activity at the concentrations tested by the three assays. Di-n-butyl sebacate, acetyl tributyl citrate and their prototype tissue conditioners were weakly to negligibly cytotoxic, while tri-n-butyl phosphate and its prototype tissue conditioner yielded the strongest cytotoxicity among the six plasticizers tested. In particular, the cytotoxicities of di-n-butyl sebacate and its prototype tissue conditioners were significantly weaker than the others and the conventional plasticizers. The results suggested that di-n-butyl sebacate and acetyl tributyl citrate could be candidate chemicals for phthalate ester-free tissue conditioners.

Key words : cytocompatibility, phthalate ester, tissue conditioner

INTRODUCTION

A number of chemicals are now understood to interfere with the steroidal regulation of normal development and function of the male and female reproductive tracts^{1–3)}. The estrogenic behaviors of certain phthalates *in vivo* and *in vitro* have been reported previously^{4,5)}. Our previous study showed that phthalate esters for dental use as plasticizers showed estrogenic activity *in vitro*⁶⁾. In addition, four commercial tissue conditioners showed estrogenic activity, and one product showed particularly strong estrogenicity⁶⁾. Hence, the development of phthalate ester-free tissue conditioners will be required.

Tissue conditioners of various types are used in linings of dentures. The type and quantity of plasticizers and other ingredients differ among materials. The physical and chemical properties of this type of material has been reported in some detail^{7–9)}. However, little information is available about their biocompatibility.

The purpose of this study was to investigate the cytocompatibility of phthalate ester-free tissue conditioners. Three candidate phthalate ester-free plasticizers, di-n-butyl sebacate (DBS), acetyl tributyl citrate (ATBC) and tri-n-butyl phosphate (TBP) are widely used as industrial chemicals and their available data are for acute toxicity, genetic toxicity, mutagenicity, carcinogenicity, reproductive toxicity and *in*

vitro toxicity¹⁰⁻¹⁵). In addition, their chemical structures have no benzene rings, which one considered to be related to estrogenicity¹⁶). First, estrogenic activities of the three candidate plasticizers were examined and then the cytotoxic effects of their plasticizers and prototype tissue conditioners made from them were measured.

MATERIALS AND METHODS

Three candidate plasticizers, DBS, ATBC and TBP, were selected and compared with three conventional plasticizers, DBP, BS and BB, which were diluted with dimethyl sulfoxide (DMSO, Lot M9H8197, Nacalai Tesque, Kyoto, Japan) (Table 1). All other chemicals were of reagent grade, obtained from commercial sources and used without further purification.

1. Estrogenic activities of the candidate plasticizers

The estrogenic activities of the three candidate plasticizers, DBS, ATBC and TBP, were measured using a yeast two-hybrid system and a fluorescence polarization system within a concentration range of 10^{-7} - 10^{-3} M.

The yeast strain used was Y190, obtained from Clontech (Palo Alto, CA, USA), and transformed with the pGBT9-receptors and pGAD424-coactivators using the lithium acetate method. An S9 hepatic microsomal fraction was used to implement the mediating influence of metabolic bioactivation. The S9 (Oriental Yeast Co., Tokyo, Japan) was prepared from rat liver induced with phenobarbital and 5, 6-benzoflavone and the cofactor ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, KCl, G-6-P, NADPH, NADH, Na_2HPO_4 and NaH_2PO_4) was dissolved in 9 ml distilled water. One milliliter of S9 was added to 9 ml of the cofactor solution, and this solution was then used as the S9 mix. Briefly, yeast transformants were grown overnight at 30°C with vigorous shaking in 1 ml of liquid synthetic medium without tryptophan and leucine. Then, 2.5 μl of the test chemicals and 25 μl of S9 mixture were added to 175 μl of fresh medium containing 50 μl of 8 hr culture in the S9 mixture group. Twenty-five micro liters of phosphate buffer solution, instead of the S9 mix, was added to the above medium in the non-S9 mixture group. After culturing for 4 hr at 30°C, the β -galactosidase activities were determined. The values were calculated as the rate of β -galactosidase activity divided by the β -galactosidase activity of 10^{-7} M 17 β -Estradiol (E2, Lot ACL1188, Wako,

Table 1 Plasticizers used in this study

Plasticizers	Code	Manufacturers	Lot
di-n-butyl sebacate	DBS	Daihachi chem ¹	N-2020
acetyl tributyl citrate	ATBC	Jplus ¹	7905
tri-n-butyl phosphate	TBP	Daihachi chem	K-20501
di-n-butyl phthalate	DBP	Nacalai Tesque ²	M9F5651
benzyl salicylate	BS	Nacalai Tesque	ZOK1028
benzyl benzoate	BB	Nacalai Tesque	MOT3521

¹Osaka Japan, ²Kyoto Japan

Osaka, Japan) used as a positive control.

For comparison, all chemicals were assessed for estrogenic activity using a fluorescence polarization system according to the technique originally described by Bolgar *et al.*¹⁷⁾ with slight modifications. The chemicals were tested for their ability to displace fluorescent non-steroid estrogen (FES) from the ER (estrogen receptor)-FES complex. A large ER-FES complex has a high anisotropy value. With increasing concentrations of a competing ligand the FES is displaced from the complex; the FES molecules are released more rapidly and therefore have a low anisotropy value. First, 50 μ l of the ER-FES complex was added to 50 μ l of screening buffer containing 1 μ l of the test chemicals, and then negative (50 μ l of FES-ER complex and 50 μ l of screening buffer, equivalent to 0% inhibition) and positive (free FES, 100 μ , equivalent to 100% inhibition) controls were measured in triplicate. After 60 min at 25°C, the anisotropy values in each tube were measured on Fluorescence Polarization Instrument (Beacon 2000, PanVera, Wisconsin, USA) with excitation at 360 nm and emission at 530 nm. Finally, the values were converted to percent inhibition.

In addition, we used MCF-7 cells in the E-screen test of estrogenicity, according to a technique originally described by Soto *et al.*¹⁸⁾, again with slight modifications. Estrogenic activities were measured within a concentration range of 10^{-9} - 10^{-4} M to avoid the cytotoxicity and estrogenicity of DMSO in the high concentration ranges. Cells, provided by Dr. Sonnenschein (Tufts University), were trypsinized, plated in 24-well plates at initial concentrations of 4×10^4 cells/ml in 5% FBS (Lot AHE8536, Hyclone, Utah, USA) in DMEM (Lot 077104, Nissui, Tokyo, Japan), and allowed to attach for 24 hr; the seeding medium was then replaced with phenol red-free DMEM (Lot 1020423, GIBCO, NY, USA) with 5% FBS (Lot AHF8529, Hyclone, Utah, USA) that had been treated to remove endogenous steroids, and different concentrations of the test chemicals were added. The assay was completed after 144 hr by removing medium from wells. The negative control was the cell culture medium including 0.1% DMSO. Cell proliferation was assessed using SRB (Sulfurhodamine - B) assay. Absorbance was measured with a microplate reader equipped with a 540 nm filter. The values measured, in triplicate, were averaged, and expressed as the fold increases over the control.

2. Cytotoxic effects of plasticizers and prototype tissue conditioners

The cytotoxic effects of DBP, BS and BB added to three candidate plasticizers were measured. Each plasticizer of 10 mM was diluted in the medium to make up 6 parallel dilution series, with dilution steps of 1 : 1.

2.1 Cytotoxicity of plasticizers to human gingival fibroblasts

Peripheral tissue, distal to a non-periodontitis involved mandibular molar of a donor undergoing surgery was retrieved after obtaining appropriate informed consent. Human gingiva fibroblasts from biopsies of attached gingiva of healthy premolar and permanent molar teeth were trypsinized, plated in 96-well plates at initial concentrations of 10^5 cells/ml in 10% FBS (Lot AHE8536, Hyclone, Utah, USA) in DMEM (Lot 077104, Nissui, Tokyo, Japan), and allowed to attach for 48 hr. The

seeding medium was replaced by medium containing different concentrations of various plasticizers with or without S9 mix. The S9 mix was prepared in the same manner described above. The negative control was the cell culture medium including 1% DMSO. After 48 hr incubation, cytotoxicity was assessed using a WST assay. Ten microliters of the Cell Count Reagent SF (Lot V9N3216, Nacalai Tesque, Kyoto, Japan) was added to each well. After incubation for 1 hr at 37°C, the absorbance of each well was measured at 450 nm with a reference wavelength at 655 nm. The values measured, in triplicate, were averaged, and expressed as percentages of the control. The IC_{50} values (the chemicals concentration required to inhibit viability by 50%) were calculated by means of nonlinear regression.

2.2 Cytotoxicity of prototype tissue conditioners for the living skin equivalent
Poly (ethyl methacrylate) (Lot 10134, Shofu, Kyoto, Japan) and six plasticizers containing ethyl alcohol at 15 wt% were used as the powder and solution, respectively. The polymer/liquid (P/L) ratio of 1.2 by weight widely employed in commercial products was utilized. Samples of the six prototype tissue conditioners were prepared in stainless steel molds (10 mm diameter \times 2 mm height), and clamped between stainless steel plates, and cured at 37°C for 5 min. An artificial human skin model that is said to mimic normal human skin tissue, TESTSKIN (Lot SS0663, TOYOBO, Osaka, Japan) possessing a 3D-structure of the epidermis over the dermis was purchased from TOYOBO and used according to the manufacturers instructions. TESTSKIN was exposed to these samples for 6 hr according to the protocol. Cytotoxicity was assessed using a WST assay. One hundred microliters of the Cell Count Reagent SF (Lot V9N3216, Nacalai Tesque, Kyoto, Japan) was added to each well. After incubation for 1 hr at 37°C, the absorbance of each well was measured at 450 nm with a reference wavelength at 655 nm. The values measured, in triplicate, were averaged, and expressed as percents of the controls.

The results are expressed as means \pm standard deviation (SD). A one-way ANOVA followed by a Tukey test was used to analyze the data. A p -value of <0.05 was regarded as significant.

RESULTS

Figs. 1 to 3 show the estrogenic activities of the three candidate plasticizers. The β -galactosidase activity above 0.1, greater than 50% inhibition and a 1.5-fold increase in cell growth over the control were considered estrogenic activity for the three respective tests, which were based on the findings of previous studies¹⁹⁾. The highest value of β -galactosidase activity among the concentrations tested was 0.03. The percent inhibition and fold increase of controls among the concentration tested did not increase beyond 20% and 1.3, respectively. As a result, none of the three plasticizers showed estrogenic activities at any concentration tested.

Fig. 4(a) and (b) show the concentration-response curve of six plasticizers with or without the S9 mixture, respectively. Table 2 shows the IC_{50} of six plasticizers with or without the S9 mixture. ATBC and DBS show a weak cytotoxicity at the

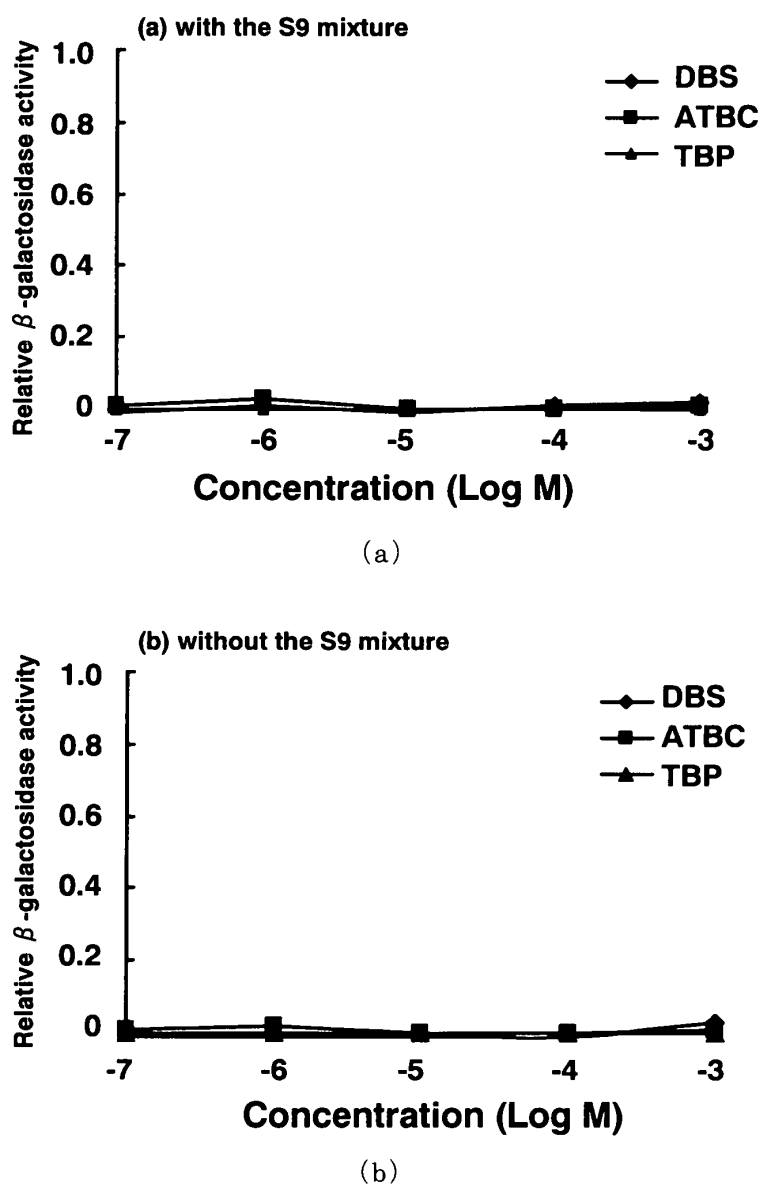


Fig. 1 Estrogenic activity of plasticizers in the yeast two-hybrid system.

Chemicals were added to yeast cultures in doses ranging from 10^{-7} to 10^{-3} M. Following 4 hr incubation, the cultures were assayed for β -galactosidase activity with or without the S9 mixture. The values are represented as the rate of β -galactosidase activity divided by the β -galactosidase activity at 10^{-7} β -estradiol. (a) with the S9 (b) without the S9 mixture

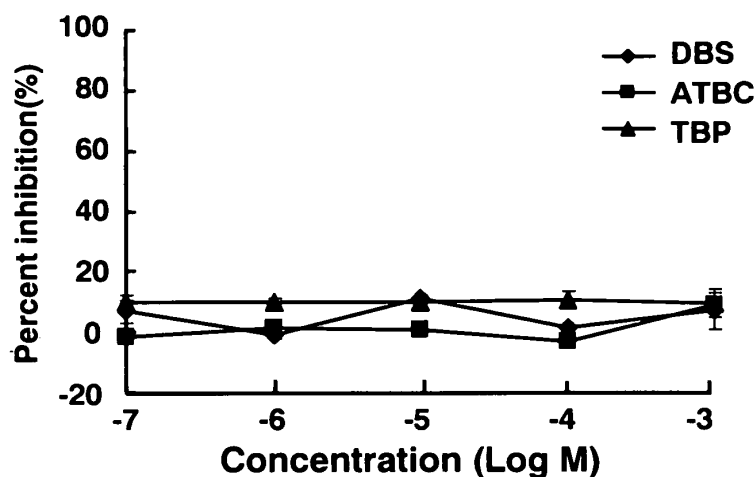


Fig. 2 Estrogenic activity of plasticizers in a fluorescence polarization system.

Chemicals were added to the screening buffer in doses ranging from 10^{-7} to 10^{-3} M with the ER-FES complex for 60 min at room temperature (25°C) followed by measurement of fluorescence polarization. Polarization findings were converted to percent inhibition. Vertical bars indicate the standard deviation.

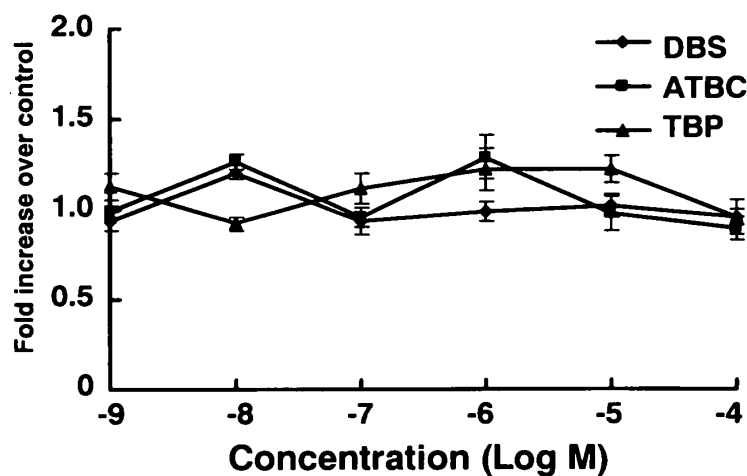


Fig. 3 Estrogenic activity of plasticizers in MCF-7 E-screen.

Chemicals were added to MCF-7 cells in doses ranging from 10^{-9} to 10^{-4} M. Following 144 hr incubation, the cultures were assayed for SRB. DMSO served as the control. The absorbance was measured with a microplate reader equipped with a 540 nm filter. The readings were averaged and the results expressed as fold increase over the control value. Vertical bars indicate the standard deviation.

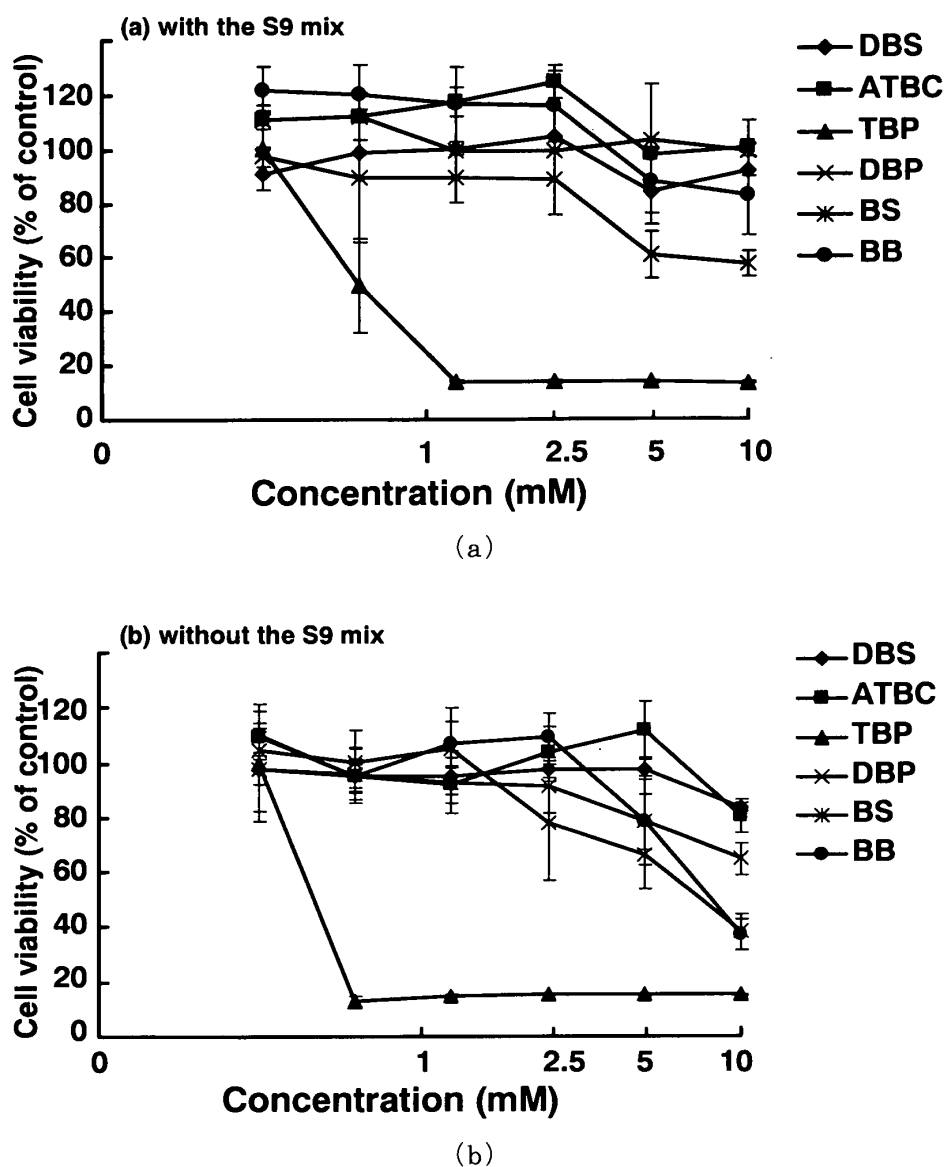


Fig. 4 Cytotoxicity of plasticizers in human gingival fibroblasts cells. Chemicals were added to human gingival fibroblasts in doses ranging from 0.3125 mM to 10 mM. Following 48 hr incubation, the cultures were assayed for WST. DMSO served as the control. The absorbance was measured with a microplate reader equipped with a 450 nm filter. The readings were averaged and the results expressed as a percent of the control value. Vertical bars indicate the standard deviation. (a) with the S9 (b) without the S9 mixture.

concentration of 10^{-3} M without S9 mixture. DBP, BS and BB showed cytotoxicity at a concentration of greater than 5 mM without S9 mixture. TBP was cytotoxic at concentrations greater than 0.625 mM without the S9 mixture. The IC_{50} s of ATBC, DBS and DBP were at concentrations greater than 10^{-3} M and those of BS, BB and

Table 2 IC₅₀ of plasticizers with or without the S9 mixture in human gingival fibroblasts cells

Plasticizers	IC ₅₀ (with the S9 mixture)	IC ₅₀ (without the S9 mixture)
DBS	10 mM<	10 mM<
ATBC	10 mM<	10 mM<
TBP	0.591 mM	0.358 mM
DBP	10 mM<	10 mM<
BS	10 mM<	5.14 mM
BB	10 mM<	9.37 mM

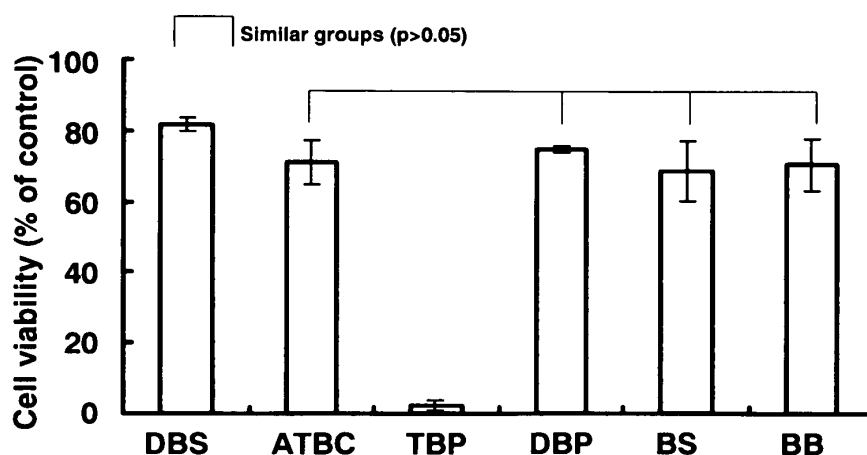


Fig. 5 Cytotoxicity of prototype tissue conditioners for living skin equivalent (LSE).

Prototype tissue conditioners exposed to living skin equivalent. Following 6 hr incubation, the cultures were assayed for WST. No sample served as the control. The absorbance was measured with a microplate reader equipped with a 450 nm filter. The readings were averaged and the results expressed as a percent of the control value. Vertical bars indicate the standard deviation.

TBP were 5.14 mM, 9.37 mM and 0.358 mM without the S9 mixture, respectively. The cytotoxicities of plasticizers with S9 mixture were considerably different from those without the S9 mixture. For example, the cell viability for 10 mM of BB with the S9 mixture was $83.0 \pm 14.9\%$, while that for without the S9 mixture was $37.0 \pm 5.62\%$. Only the IC₅₀ of TBP was determined; the others were at concentrations greater than 10 mM with S9 mixture.

Fig. 5 shows the cytotoxicity of the prototype tissue conditioners made from six plasticizers. DBS showed the weakest cytotoxicity among those tested (81.7 ± 1.94 , $P < 0.05$). There were no significant differences among BS, DBP, ATBC and BB (68.8 ± 8.72 , 75.2 ± 0.93 , 71.3 ± 6.11 and 70.6 ± 7.40). On the other hand, TBP showed the strongest cytotoxicity among those tested (2.36 ± 1.58 , $P < 0.01$).

DISCUSSION

DBS, ATBC and TBP have been chosen as alternative plasticizers of phthalate ester-free tissue conditioners. ATBC and DBS are plasticizers found in polyvinylidene-chloride film used for packing food^{10,14}. TBP is used in aircraft hydraulic fluid, herbicides, coating, inks and in an oil-well drilling defoamer¹¹. However, there is little information available on the cytocompatibility of their plasticizers. None of the three candidate plasticizers tested were estrogenic in the three *in vitro* assays. DBS and ATBC and prototype tissue conditioners made from them were weakly to negligibly cytotoxic among the six plasticizers tested in human gingival fibroblasts and the living skin equivalent, respectively.

We measured the estrogenic activities of these plasticizers using the yeast two-hybrid system assay, competition binding assay and E-screen assay. These assays have been widely applied to identify estrogenic chemicals and to determine the relative potencies for hormonal responses. Andersen *et al.*²⁰ suggested that although there is general agreement among the above three assays, there are certain performance characteristics and sources of error that should be considered in the use of the assays, either alone or in combination. The yeast two-hybrid system is based on the ligand-dependent interaction between a nuclear hormone receptor and a coactivator. Using the activation domain derived from the yeast transactivator GAL4, strong β -galactosidase activity could be obtained²¹. In the present study, the estrogenic activities of metabolites were also measured in the presence of the rat liver S9 mixture. The liver is the main organ for the metabolism of fractions, such as the fraction from the livers of rats treated with inducers of mixed-function oxidase activity, and has been widely employed as the xenobiotic activating system *in vitro* cytotoxicity assay²². Our previous study²³ demonstrated that bisphenol-A (BPA) and bisphenol-A-related chemicals showed estrogenic activities, and enhanced them in the presence of the rat liver S9 mixture. On the other hand, Nakagawa *et al.*²⁴ showed that the estrogenic activity of metabolites of BPA, 3-hydroxybisphenol-A, was less than that of BPA in MCF-7 E-screen assay and competing binding assay. Consequently, three plasticizers, which have not been shown to exhibit β -galactosidase activity at the concentrations tested with or without the S9 mixture were inactive or undetectable with the yeast two-hybrid system.

For comparison, we adopted an estrogen/ER competition binding assay and used it for the rapid screening of endocrine disrupting chemicals for ER binding activity. This assay uses fluorescence polarization to monitor the displacement of a high-affinity fluorescent ligand from purified recombinant human ER¹⁷. Furthermore, this assay can be run at physiological temperatures, requires less than half a day to complete, and involves no radioactivity¹⁷. BPA and BPA-related chemicals showed estrogenic activity in our previous study^{19,23,25}, but phthalate esters other than butyl benzyl phthalate did not. The three plasticizers were not shown to have binding interactions with the ER, and, as a result were inactive.

We also used the E-screen test described by Soto *et al.*¹⁸. This assay compares

the cell yield between cultures of breast tumor-derived MCF-7 cells treated with estradiol and cultures treated with different chemicals. The reported detection limit of 10 pg E2/ml makes the MCF-7 cell E-screen one of the most sensitive assays for assessing the estrogenicity²⁶⁾. Only this assay among the three assays have detected estrogenicity of the plasticizers employed as dental materials in the previous study⁶⁾. Despite the high sensitivity of this assay in detecting estrogenicity, it also has a limitation. When a chemical was cytotoxic to MCF-7 cells, the E-screen could not be used. The three plasticizers tested in this study did not increase the fold increase of control. Hence, it was not possible to reproduce the estrogenic activity in their plasticizers.

It was reported that there is a relationship between the structures of a chemical and its estrogenic behavior¹⁶⁾. In general, estrogenicity is related to chemical structures bearing benzene rings¹⁶⁾. In our previous study¹⁹⁾, methyl methacrylate, hydroxyethyl methacrylate and triethylenglycol dimethacrylate etc., which have no benzene rings, did not show estrogenic activity among the dental materials tested. On the other hand, bisphenol-A-related diphenylalkanes and phthalate esters with benzene structures did^{19,23,25)}. In exceptional cases, non-ring structural di-2-ethylhexyl adipate and chlormequat chloride were estrogenic by measuring the direct binding of the chemical to the fish estrogen receptor and MCF-7 E-screen assay, respectively^{20,27)}. In the present study, the three candidate plasticizers were inactive since DBS, ATBC and TBP, which do not have any benzene rings, were not estrogenic.

In this study, an *in vitro* model of human gingival fibroblasts was used. Primary cell cultures are considered to retain characterization of target tissue cells well in comparison to permanent cell lines. On the other hand, Geurtsen *et al.*²⁸⁾ reported that cytotoxicity tests with primary cells must be considered reproductions owing to the derivation from various donors and characterization by a finite life span. However, it was found by Lehmann *et al.*²⁹⁾ that the above tests were reproducible when cells from passages 4 to 8 had been used. The cytotoxic effect was concentration-dependent, as expected. DBP, BS and BB yielded moderate, and TBP strong cytotoxicity, respectively, while DBS and ATBC did not. Similar observations were reported by Ekwall *et al.*¹⁰⁾, who reported that phthalates and sebacates were not toxic to the HeLa cells, while the phosphates had strong cytotoxicity by the MIT-24 test system. At the same time, they showed strong cytotoxicity of citrates. The present results were similar to those reported by Mochida *et al.*¹³⁾ where ATBC and DBS showed similar cytotoxicity results using human KB cells, monkey Vero cells and canine MDCK cells by determining the extent of growth inhibition. The toxicity of TBP *in vivo* has been investigated¹¹⁾. A high dosage in rats caused coma, dyspnea and pulmonary edema. Arnold *et al.*¹²⁾ reported that TBP at high doses produced tumors of the bladder in rats. On the other hand, negative results were reported in *in vitro* or *in vivo* tests for mutagenicity and genotoxicity¹¹⁾. BB and BS have been used as fragrance and flavor ingredients. Kohrman *et al.*³⁰⁾ reported that BS has a very low potential to induce hypersensitivity or to elicit reactions that are presumably attributable to pre-existing sensitization. The cytotoxicities of metabolites of six

plasticizers were evaluated in the present study. The cell viabilities of TBP, DBP BS and BB were reduced in the presence of the S9 mixture. Hikage *et al.*²²⁾ reported that dental monomers in the presence of the S9 mixture reduced cytotoxicity in JTC-12 cells, and suggested that the monomers might be metabolized to lower toxic metabolites by the S9 mixture. This might be expected because these plasticizers appear to be metabolized following oral exposure, and therefore the metabolites are more likely to be less cytotoxic. The primary metabolite of dibutylphthalate is monobutylphthalate, and differences of cell behaviors were reported between monophthalate and di-n-phthalate³¹⁾. However, we did not identify metabolites by the S9 mixture of the plasticizers tested in this study. Engelmann *et al.*,³²⁾ showed that NMR spectroscopy of cell-material interactions might reveal metabolic effects of organic test substances, which are not detectable by standard *in vitro* assays. Further analysis of these metabolites will be required.

A three-dimensional human skin model aiming at morphology of native epidermis has been developed and used to evaluate skin irritancy caused by chemical reagents, cosmetics, medicines, etc³³⁾. We used this to examine the cytotoxicity of the prototype tissue conditioners made from six plasticizers. An organotypic co-culture of human dermal fibroblasts in a collagen-containing matrix, this living skin equivalent, has fibroblasts and keratinocytes as its viable and metabolically active cell population³⁴⁾. In normal oral mucosa, these cells usually exist in the oral epithelium and lamina propria. Thus, the present assay can be regarded as being more strict than *in vivo* testing. DBS and TBP showed the weakest and strongest cytotoxicities in the 2-D culture, respectively. A similar tendency was observed in the cytotoxicity of prototype tissue conditioners made from them in the 3-D culture. However, the other four plasticizers showed different tendencies between the 2-D and the 3-D cultures. This suggests that the cytotoxicity of tissue conditioners might be influenced by one of the plasticizers. On the other hand, the keratin layer could function as a strong barrier to interfere with reagent permeation into the collagen-containing matrix. In this model, Kayama *et al.*³⁵⁾ reported that unset cold-curing acrylic resins containing tissue conditioners showed strong cytotoxicity due to high reagent permeation. Watanabe *et al.*³⁶⁾ showed that the diffusion parameters of all drugs used were not different in an experiment using living skin equivalent, whereas the barrier function of living skin equivalent is much lower than those of human and rat skin.

Ethanol and plasticizers are considered to be the two main components that will leach from tissue conditioners. The change in elasticity due to dissolution of the two components is a key function of these dental materials, which are used to condition damaged edentulous soft tissues. Therefore, the biocompatibility of plasticizers should be elucidated. Recently, transgenerational studies on the developmental reproductive toxicities of phthalates have demonstrated that several of these produce malformations in male rat offspring after *in utero* and neonatal treatment^{2,37,38)}. Mylchreest *et al.*³⁸⁾ identified a NOAEL of 50 mg/kg/d for DBP. Sharpe *et al.*²⁾ showed that the exposure of male rats to BBP had adverse effects on rat testes size and sperm production. An *in vitro* study^{6,20,31)} showed that a small number of the

phthalates had extremely weak estrogenicity in the recombinant yeast screen and MCF-7 E-screen. More recently, the estrogenicities of plasticizers other than phthalate esters were also reported⁶⁾. In another aspect of biocompatibility, it was reported that oral diseases such as denture stomatitis and burning mouth syndrome could be associated with compounds leaching from dentures³⁹⁾. Okita *et al.*⁴⁰⁾ showed that the tissue conditioners tested appeared to be more cytotoxic than autopolymerizing denture bases using the agar overlay method.

Additional studies will be necessary to investigate the cytocompatibilities of phthalate ester-free tissue conditioners. In general, mixtures of more than two plasticizers have been used as the liquid components of commercial tissue conditioner. The possibility of biologically synergetic effects must be considered. In the biocompatibility testing of the palladium alloy casting, Syverud *et al.*⁴¹⁾ reported that the mixture $\text{Pd}^{2+} + \text{Cu}^{2+}$ released from the alloys was the most irritating to mucous membranes as evaluated by the HET-CAM method. In the synergetic effects for estrogenicity, Harris *et al.*³¹⁾ measured the estrogenic activity of the active phthalate alone and in combination with 17β -estradiol, and in no case was the response significantly greater than predicted if additivity had occurred. Another expected study is to investigate the effects of these plasticizers on the growth of *Candida*. Nikawa *et al.*⁴²⁾ demonstrated that two plasticizers, BS and BB significantly decreased the growth rate of *Candida*. These two plasticizers were moderately toxic to the human fibroblasts in this study. More recently, Lefebvre *et al.*⁴³⁾ showed that the addition of Microban, an introduced additive containing triclosan, provided resistance to growth of bacteria and fungi but did not significantly alter the cytotoxicity of the denture lining material or reduce the adherence of viable *Candida albicans* to the surface of the denture lining material. Moreover, leaching of plasticizers from tissue conditioners has been a concern as described above. We measured the plasticizers that leached from the prototype tissue conditioners (data not shown). Phthalate esters could be measured, but, the three candidate plasticizers could not. Their plasticizers, because of their non-ring structure, were not detected by UV wavelengths in HPLC. Other detection methods must be employed to clarify leaching from phthalate-free tissue conditioners.

In summary, three candidate plasticizers, DBS, ATBC and TBP were not estrogenic in this study. The prototype tissue conditioners made from DBS and ATBC showed less cytotoxicity than from phthalate esters. The findings from the current study suggested that phthalate ester-free prototype tissue conditioners would be more cytocompatible than the conventional commercial tissue conditioners. More recently, we demonstrated⁴⁴⁾ that the prototypes made from DBS and ATBC have sufficient physical properties as tissue conditioners. Thus, this new material appears to have potential as an alternative to conventional tissue conditioners. However, *in vitro* studies cannot be directly extrapolated to *in vivo* conditions, and further studies will be required to examine the accumulation, metabolism and excretion of these chemicals after they have been taken into the body orally. In addition, information on various aspects of materials science could lead to the development of new

phthalate ester-free tissue conditioners.

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