Estrogenic activity of chemicals for dental and similar use *in vitro*

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The estrogenic activities of chemicals for dental and similar use were tested by a reporter gene assay (yeast two-hybrid system) and an estrogen/estrogen receptor (ER-α) competition binding assay (fluorescence polarization system). Among the 10 chemicals [bisphenol-A (BPA), bis-2-hydroxypropyl methacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA), methyl methacrylate (MMA) and 2-hydroxyethyl methacrylate (HEMA), dibutyl phthalate (DBP), n-butyl benzyl phthalate (BBP), n-butyl phthalyl n-butyl glycolate (BPBG), di-2-ethylhexyl phthalate (DEHP), and di-2-ethylhexyl adipate (DOA)], which were diluted with DMSO to concentrations ranging from 5×10^{-7} to 5×10^{-3} M, and 17β-estradiol (E2) as a positive control, BPA and BBP showed estrogenic activity in these two assays, while the remaining eight chemicals did not at the concentrations tested. Additional data, together with *in vivo* and epidemiological examinations, are required. Such investigations should also provide information on the validity of these methods for testing the estrogenic activity of chemicals.

1. Introduction

Advanced materials have greatly contributed to improvements in dental treatment and to the maintenance of dental health. However, while they enable us to meet patients' needs, they might also have adverse effects such as hypersensitivity. Elution of the chemical BPA from a commercial fissure sealant was reported by Olea *et al*. [1], which raised a controversy over the sealant's use as a dental material. The industrial chemical BPA has been shown to have estrogenic activity *in vivo* and *in vitro* [2, 3]. Another group of chemicals, phthalates, which have been widely used in the chemical and health industries, have also been implicated as environmental estrogens. Phthalate esters are used in denture lining and tissue-conditioning materials for dental treatment. Other sources in foods, the air, and the environment are suspected to be estrogen mimics, and may present a health hazard to humans. In view of this uncertain situation, it is necessary to determine whether chemicals for dental use have estrogenic activity. The purpose of the present study was to examine the estrogenic activities of chemicals for dental and similar use by a reporter gene assay (yeast two-hybrid system) and an estrogen/estrogen receptor (ER-α) competition binding assay (fluorescence polarization system).

2. Materials and methods

The chemicals shown in Table I were diluted with DMSO to concentrations ranging from 5×10^{-7} M to 5×10^{-3} M. 17β-estradiol (E2) at 10^{-7}M was used as a positive control. All other chemicals were reagent grade, obtained from commercial sources, and used without further purification.

We used the yeast two-hybrid system to examine the estrogenic activity of the chemicals according to a technique originally described by Nishikawa *et al*. [4]. Briefly, yeast transformants were grown overnight at 30 °C with vigorous shaking in 1 ml of selective medium. Fifty μl of the overnight culture was then added to 200 μl of fresh medium containing the test chemicals. After the yeasts were cultured for 4 h at 30 °C, β-galactosidase activities were determined. For comparison, all chemicals were assessed for estrogenic activity using a fluorescence polarization system according to the

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TABLE I Materials used in this study

Chemicals	Purity	Manufacturers	Lot
bisphenol-A (BPA)	> 99%	Tokyo Kasei	GG01-DK
bis-2-hydroxypropyl methacrylate (Bis-GMA)	> 99%	Polyscience	470354
triethylene glycol dimethacrylate (TEGDMA)	> 95%	Wako	SKH4994
methyl methacrylate (MMA)	> 98%	Wako	DLG6719
2-hydroxyethyl methacrylate (HEMA)	> 95%	Wako	WTQ4257
dibutyl phthalate (DBP)	> 98%	Wako	ACA4421
n-butyl benzyl phthalate (BBP)	> 97%	Nacalai Tesque	M8K5328
n-butyl phthalyl n-butyl glycolate (BPBG)	> 93%	Tokyo Kasei	GC01
di-2-ethylhexyl phthalate (DEHP)	> 99%	Wako	ACK8694
di-2-ethylhexyl adipate (DOA)	> 99%	Wako	TPM7602

technique originally described by Bolgar *et al*. [5] with slight modifications. Briefly, the 10 chemicals were tested for their ability to displace the fluorescent ligand ES1 from the ER-ES1 complex. Fifty μl of ER-ES1 complex was added to 50 μl of screening buffer containing 1 μl of the test chemicals. Negative (the fluorescent ligand ES1 100 μl) and positive (50 μl of ES1-ER complex and 50 μl of screening buffer) findings in the absence of a competitor were measured in triplicate. After 60 min at 25 °C, the anisotropy values in each tube were measured on a Beacon 2000 Fluorescence Polarization Instrument (PanVera Corporation) with excitation at 360 nm and emission at 530 nm. Finally, the anisotropy values were converted to percent inhibition.

3. Result

The relative β-galactosidase activities of the test chemicals in the two-hybrid system are shown in Fig. 1. BPA induced β-galactosidase activity at concentrations of 5×10^{-5} M and 5×10^{-4} M, and BBP induced such activity at concentrations of 5×10^{-5} M and 5×10^{-4} M. However, the remaining eight chemicals did not induce β-galactosidase activity at the concentrations tested. BPA and BBP did not induce β-galactosidase activity at the highest concentration of 5×10^{-3} M.

The percent inhibition findings for the test chemicals in the fluorescence polarization system are shown in Fig. 2. BPA increased the percent inhibition at 5×10^{-5} M and above, while BBP increased the percent inhibition at 5×10^{-5} M and above. The remaining eight chemicals did not have similar effects at the concentrations tested.

4. Discussion

In this paper, the estrogenic activity of chemicals for dental and similar use was tested by a reporter gene assay and an estrogen/estrogen receptor (ER-α) competition binding assay. BPA and BBP showed estrogenic activity by these two assays, but the remaining eight chemicals did not at the concentrations tested.

The ability of certain endocrine-disrupting chemicals to mimic the effects of natural steroid hormones and their potential to disrupt the delicate balance of the endocrine system in animals and humans are of increasing concern. Many experiments have documented that various environmental chemicals can act as endocrine disrupters, which clearly shows the estrogenic activity of some environmental chemicals. When new materials are developed, various aspects of toxicity are examined, followed by clinical examinations based on informed consent. However, estrogenic activity is not among the factors tested. Polymers are used in important applications in dental treatment, such as restorative materials, liners, adhesives, oral prosthetic devices, tissue substitutes and rebase materials. Bis-2-hydroxypropyl methacrylate (Bis-GMA) and some other monomers like triethylene glycol dimethacrylate (TEGDMA), 2-hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA) are widely used. Monomers consisting of large molecules have several component monomers, the most notable of which is BPA in the case of Bis-GMA. There have been several studies on their effects on pulpal injury and their cytotoxicity. Stanley *et al*. [6] reported that most of the eight individual components of composites failed to cause significant necrosis inflammation when placed for 21 days on pulpal dentin in monkey teeth. In a study of the cytotoxicity of resins, the cytotoxic concentrations of 11 components of composites were reported by Hanks *et al*. [7] They found that the ID50 concentrations for Bis-GMA, TEGDMA and BPA ranged between 10^{-5} and 10^{-4} M, and BPA was the least toxic of the three monomers. Yoshii *et al*. [8] examined the relationship between the structure and cytotoxicity of monomers used in dental resin materials, and reported that the cytotoxicity ranking of monomers was Bis-GMA > TEGDMA > HEMA > MMA. In a related incident, a dental assistant developed allergic contact dermatitis caused by BPA containing composites based on epoxy dimethacrylate [9]. However, the estrogenicity of dental materials was not noted until Olea *et al*. identified its presence in 1996 [1].

BPA showed estrogenic activity in the present study. BPA induced β-galactosidase activity at concentrations of 5×10^{-5} M and 5×10^{-4} M and increased the percent inhibition at 5×10^{-5} M and over. The estrogenic activity in this assay is comparable to that reported by Villalobos *et al*. [10], but seems to be lower than that reported by Olea *et al*. [1] for the E-screen. E-screen was originally proposed to be used to determine whether a substance is an estrogen. Villalobos *et al*. [10] pointed out that since the E-screen assay was based on the ability of MCF7 cells to proliferate in the presence of estrogens, differences in sensitivity to estrogen between MCF-7 and other cells could lead to different results. Differences between *in vitro* assay methods might also lead to

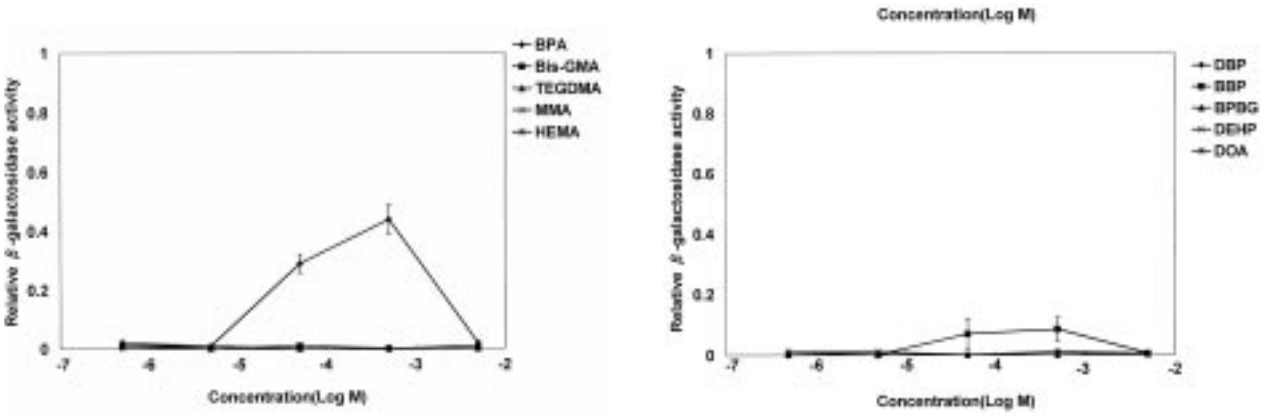

Figure 1 Dose-response curves of chemicals for dental for a variety of chemicals in use and similar. Chemicals were added to yeast cultures in doses ranging from 5×10^{-7} to 5×10^{-3} M. Following 4 h incubation the cultures were assayed for β-galactosidase activity. The values are represented as the rate of β-galactosidase activity divided by the β-galactosidase activity by 1×10^{-7} M 17β-estradiol. The error bars except for BPA are too small and are therefore not shown on the figure.

different results. Bis-GMA did not show estrogenic activity in this study. The diphenylalkanes which have two terminal hydroxyl groups were assayed in the para position, either free or as ether or ester bonds [11]. Ester derivatives of diphenylalkanes were estrogenic, in contrast to some ether substituents at the terminal -OH, such as Bis-GMA which showed no estrogenic activity in the E-screen [11]. However, more recently, Nathanson [12] reported from an *in vitro* study that Bis-GMA significantly increased MCF-7 cell growth at 10^{-8} M and had an estrogenic effect. Moreover, Mariotti *et al*. [13] reported that short exposure to Bis-GMA was not sufficient to cause any consequential changes in sex accessory tissues. MMA, HEMA and TEGDMA did not show estrogenic activity in this study. The present results are consistent with the fact that compounds containing benzene rings have estrogenic activity [14], since the three chemicals without benzene rings in their structures did not show estrogenic activity.

Many industrial and consumer polyvinyl chloride-type products generally contain one of the phthalate esters as a plasticizer. These products include industrial tubes, transportation vehicles, floor tiles and numerous other products. Polyvinyl chloride is also widely used in the manufacture of medical items such as medical tubing, catheters, blood containers and coatings for drugs. DEHP is produced in extremely large volumes, and is the most widely used plasticizer in Japan today, while BBP is used by industry in smaller quantities. DEHP was found to be leached into blood from plastic tubing and from plastic bags used for blood storage [15]. While in general the acute toxicity of the most commonly encountered phthalates is low, some phthalates have been shown to be carcinogenic, to result in liver poisoning, and to cause reproductive toxicity at higher doses [16–18]. Specifically, several phthalate esters including DEHP and DBP are male reproductive toxicants in rats and mice. An *in vivo* study has demonstrated that BBP has adverse effects on the size of rat testis and sperm production [19]. Some phthalates have been used as a softening agent to obtain plasticity of dental materials, with a typical application being denture liner. This consists of a powder of polyethyl methacrylate and a solvent such as ethyl alcohol and aromatic ester. The alcohol and plasticizer will leach rapidly from the mixture as the plasticity of the material decreases. Kawaguchi *et al*. [20] reported that phthalate esters (DBP and BPBG) leached from denture liners into water rapidly for the first week, and this leaching then gradually subsided. Lower amounts of phthalate esters were still detected after immersion in water for 2 weeks. BPBG is also used as a plasticizer for denture liners.

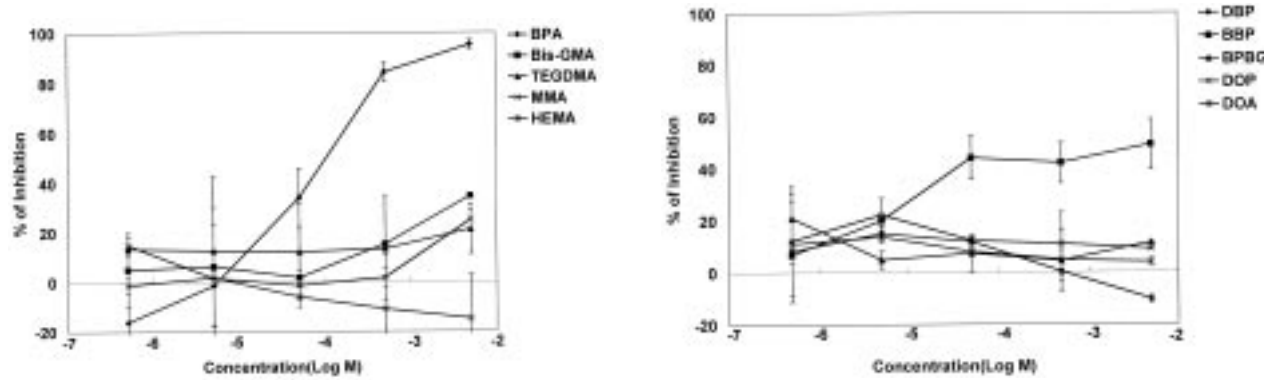

Figure 2 Competition binding curves for a variety of chemicals in dental use and similar against a human recombinant estrogen receptor α/fluorescent ligand complex (ER-ES1 complex). Chemicals were added to the screening buffer in doses ranging from 5×10^{-7} to 5×10^{-3} M with ER-ES1 complex for 60 min at room temperature (25 °C) followed by measurement of fluorescence polarization. Polarization data was converted to percent inhibition.

Among the five phthalates examined, only BBP showed weak estrogenic activity, while the others showed none. Harris *et al*. [21] reported that BBP and DBP showed weak estrogenic activity using a recombinant yeast screen for mitogenic effects on estrogen-responsive human breast cancer cells, but DEHP had no effect. DOA was the only adipic acid used as a test chemical in this study, and is also listed as a chemical that is associated with endocrine system effects in animals and humans and *in vitro* by the Illinois Environmental Protection agency [22]. Jubling *et al*. [23] reported that BBP, DBP, DEHP and DOA were estrogenic by measuring the direct binding of these chemicals to the fish estrogen receptor, but BBP and DBP were estrogenic using mammalian estrogen screens *in vitro*, while DEHP and DOA were not. Nishikawa *et al*. [4] reported that the activity of β-galactosidase could be enhanced by an overnight incubation in the two-hybrid system. The reason for the discrepancy among the reports is not clear, but it seems to be due in part to the sensitivities of the respective assays.

The yeast two-hybrid system is an effective method for examining the interaction between hormone receptors and coactivators to assess the potential estrogenic activities of chemicals. The fluorescence polarization system, which measures the capacity of a competitor chemical to displace a high-affinity fluorescent ligand from purified, recombinant human ER-α, is also useful. These systems are beneficial because of their specificity, sensitivity, rapidity and simplicity. It has been shown that a reporter gene assay and an estrogen/estrogen receptor (ER-α) competition binding assay (fluorescence polarization system) are practical for use as large-scale screening tools to characterize the estrogenic activity of chemicals. Despite the usefulness of these methods in detecting estrogenicity, they also have limitations. For example, BPA and BBP did not induce galactosidase activity at the highest concentration of 5×10^{-3} M. Furthermore, when a chemical was toxic against yeast, the yeast assay could not be used. Since this assay is based on the value of the absorbance after 4 h of incubation with viable yeast, the yeast would be damaged if the chemical was toxic. This would naturally lead to a decrease or complete loss of absorbance. This may be the case for BPA and BBP at a concentration of 5×10^{-3} M. In the fluorescence polarization system, a decrease in the percent inhibition for Bis-GMA at concentrations higher than 5×10^{-3} M was observed. Although the reasons for this result are not clear, precipitation of the screening buffer occurred at this concentration. This phenomenon, or staining by chemicals, might interfere with the system.

In conclusion, two of the chemicals tested by the two assay systems were estrogenic. However, it is not sufficient merely to determine that they are estrogenic. Additional data, together with *in vivo* and epidemiological examinations, are required. Since the chemicals we tested did not have any common structural features, there is no obvious part of their molecular structure that clearly enables binding to the estrogen receptor. Therefore, a molecule's structure and its estrogenicity are important aspects to be elucidated from the viewpoint of materials science. Furthermore, elution of the chemicals which were demonstrated to be estrogenic must be examined under physiologic and clinical conditions. The accumulation of relevant information on various aspects of materials science and estrogenicity could lead to a possible reconsideration of these chemicals in dental use.

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