Estrogenic Activity of Dental Materials and Bisphenol-A Related Chemicals *in vitro*

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Twenty-eight chemicals used as dental materials and bisphenol-A related chemicals were diluted with DMSO to concentrations ranging from 10^{-7} to 10^{-3} M and tested for estrogenicity. Bisphenol-A (BPA), bisphenol-F (BPF) and bisphenol-A-bischloroformate (BPACF) showed estrogenic activity using the yeast two-hybrid system, and BPA, BPF, BPACF and bisphenol-S (BPS) showed estrogenic activity using the fluorescence polarization system. However, none of the remaining chemicals and none of the dental materials showed any activity at concentrations between 10^{-7} and 10^{-3} M. Although BPA, BPF, BPACF, bisphenol-A-dimethacrylate and BPS showed estrogenic activity in the E-screen test, the remaining chemicals did not. Thus, most of the chemicals showed consistent results, either positive or negative, by the three testing methods, while two chemicals showed conflicting results. Further studies, together with *in vivo* and epidemiological examinations, are required. Elucidation of the structure-activity relationships of these chemicals is also needed to estimate the estrogenicity of a chemical from its structure.

Key words : Estrogenic activity, Bisphenol-A, *in vitro*

INTRODUCTION

A significant number of compounds with diverse chemical structures reportedly have estrogenic activity^{1–3)}. The chemical structure of one class of endocrine disrupting chemicals that mimic estrogens is known to include two phenolic rings joined through a bridging carbon. These diphenylalkanes have the generic name bisphenols: bisphenol-A (BPA) when the bridging group contains two methyl groups¹⁾.

One of the main resin components of dental composites contains 2,2-bis [4-(2-hydroxy-3-methacryloxypropoxy)phenyl] propane (Bis-GMA), may be synthesized from BPA and glycidyl methacrylate or, alternately, from the diglycidyl ether of bisphenol-A and methacrylic acid⁴⁾, where the latter process has been adopted by a number of manufacturers. Different manufacturing processes could result in different purity levels of Bis-GMA⁵⁾. These chemicals, as well as polymers with many different chemical structures, have important applications in dental treatment, *i.e.* as restorative materials, liners, adhesives, oral prosthetic devices, tissue substitutes and rebase materials.

It is necessary to determine whether chemicals for dental use have estrogenic activity. *In vitro* tests have potential utility for the rapid identification of suspect compounds. Several *in vitro* assays for measuring estrogen, androgen and dioxin like activity of compounds have been developed. These assays use a variety of end-points,

including enzyme and gene induction, ligand binding, increased protein expression and cell proliferation and differentiation⁶). No assay has yet been considered as a reliable *in vitro* testing method. Further validation processes are needed. Considering the above situation, it is wise to choose candidate methods. The purpose of the present study was to examine the estrogenic activities of dental materials and BPA-related chemicals by three *in vitro* bioassays based on different principles.

MATERIALS AND METHODS

The chemicals shown in Table 1 were diluted with DMSO (Lot M9H8197, Nacalai Tesque, Kyoto, Japan) to concentrations ranging from 10^{-7} to 10^{-3} M. 17β -Estradiol (E2, Lot ACL1188, Wako, Osaka, Japan) 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.*⁷) Briefly,

Table 1 Dental materials and bisphenol-A related chemicals used in this study

Materials	Code	Manufacturers	Batch
Methyl Methacrylate	MMA	Wako ¹	DLG6719
2-Hydroxyethyl methacrylate	HEMA	Wako	WTQ4257
2-Methacryloyloxyethyl phenyl phosphate	Phenyl-P	Kuraray ²	PP-000853
4-Methacryloyloxyethyl trimeritic acid	4-META	Sun Medical ³	97002
4-Methacryloyloxyethyl trimeritic acid anhydrate	4-MET	Sun Medical	98072
4-Acryloyloxyethyl trimeritic acid	4-AETA	Shofu ⁴	EF018
4-Acryloyloxyethyl trimeritic acid anhydrate	4-AET	Shofu	GJ093
Trimethylolpropane Trimethacrylate	TMPTMA	Shinnakamura Chem ⁵	0627X
Trimethylolpropane Triacrylate	TMPTA	Shinnakamura Chem	0730K
Hydroquinone	HQ	Tokyo KASEI ⁶	M33156K
Camphorquinone	CQ	Kishida Chem ⁷	FCW01
Monoethoxylene glycol dimethacrylate	1G	Shinnakamura Chem	0223K
Diethoxylene glycol dimethacrylate	2G	Shinnakamura Chem	0730K
Triethoxylene glycol dimethacrylate	3G	Shinnakamura Chem	SKH4944
Urethane dimethacrylate	UDMA	Shofu	50218
Neophentylglycol dimethacrylate	NPG	Shinnakamura Chem	0515K
Bisphenol-A diglycidylether dimethacrylate	Bis-GMA	Polyscience ⁸	470354
Bisphenol-A-dimethacrylate	Bis-DMA	Aldrich Chem ⁹	06430DG
Bisphenol-A methacryloyloxyethoxy phenyl propane	Bis-MEPP	Shinnakamura Chem	0410K
Bisphenol-A methacryloyloxypolyethoxy phenyl propane	Bis-MPEPP	Shinnakamura Chem	0824X
Bisphenol-A	BPA	Tokyo KASEI	GG01-DK
Bisphenol-F	BPF	Aldrich Chem	00125KJ
Bisphenol-A-bischloroformate	BPACF	Aldrich Chem	16424LS
Bisphenol-A-proxylate	P-BPA	Aldrich Chem	12802HR
Bisphenol-A-ethoxylate	E-BPA	Aldrich Chem	05104EF
Bisphenol-A-ethoxylate diacrylate	BPAEDA	Aldrich Chem	12802HR
Bisphenol-A-diglycidyl ether	BPDGE	Aldrich Chem	12925DR
Bisphenol-S	BPS	Tokyo KASEI	GG01

¹Osaka, Japan, ²Osaka, Japan, ³Moriyama, Japan, ⁴Kyoto, Japan, ⁵Wakayama, Japan,

⁶Tokyo, Japan, ⁷Osaka, Japan, ⁸Illinois, USA, ⁹Milwaukee, USA

Fig. 1 Chemical structures of dental materials and bisphenol-A related chemicals used in this study.

yeast transformants were grown overnight at 30°C with vigorous shaking in 1 ml of selective medium. Fifty microliters of the overnight culture was then added to 200 μ l of fresh medium containing the test chemicals. After the yeasts were cultured for 4 hr at 30°C, the β -galactosidase activities were determined. The values are presented as the rate of β -galactosidase activity divided by that of 10^{-7} M 17β -estradiol. For comparison, all chemicals were assessed for estrogenic activity using a fluorescence polarization system according to the technique originally described by Bolger *et al.*⁸⁾ with slight modifications. The chemicals were tested for their ability to displace the fluorescent ligand ES1 from the ER-ES1 complex. First, 50 μ l of the ER-ES1 complex was added to 50 μ l of screening buffer containing 1 μ l of the test chemicals, and then negative (the fluorescent ligand ES1 100 μ l) and positive (50 μ l of the ES1-ER complex and 50 μ l of screening buffer) findings in the absence of a competitor were measured in triplicate. After 60 min at 25°C, anisotropy values in each tube were measured on a Beacon 2000 Fluorescence Polarization Instrument (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 Villalobos *et al.*⁹⁾, again with slight modifications. Cells, purchased from the American Type Culture Collection (ATCC), were trypsinized, plated in 96-well plates at initial concentrations of 4×10^4 cells/ml in 10% FBS (Lot AGD6411, Hyclone, Utah, USA) in DMEM (Lot 07591141, Nissui, Tokyo, Japan), and allowed to attach for 24 hr; the seeding medium was then replaced with phenol red-free DMEM (Lot 1056152, GIBCO, NY, USA) with 10% 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. DMSO served as a control. NR-medium was added to each well, which was then incubated for 3 hr. The staining technique was as described by Borenfreund *et al.*¹⁰⁾ Absorbance was measured with a microplate reader equipped with a 540 nm filter. The values measured, usually in quadruplicate, were averaged, and expressed as the fold increase over the control.

The significance of differences between the different treatment groups was assessed by ANOVA and Scheffe's test. A *p*-value of <0.05 was considered significant.

RESULTS

The relative β -galactosidase activities of the test chemicals in the two-hybrid system are shown in Figs. 2 a-d. BPA and BPF induced β -galactosidase activity at concentrations of 10^{-4} and 10^{-3} M. The highest activity was observed by BPF (0.66 ± 0.18) among the chemicals that induced β -galactosidase activity at 10^{-3} M ($p < 0.01$). BPACF induced β -galactosidase activity at a concentration of 10^{-4} M. However, the remaining 25 chemicals did not induce β -galactosidase activity at the concentrations tested.

The percent inhibition findings for the test chemicals in the fluorescence polarization system are shown in Figs. 3 a-d. BPA and BPF increased the percent inhibition

Fig. 2 a

Fig. 2 b

Fig. 2 c

Fig. 2 d

Fig. 2 Dose-response curves of chemicals. Chemicals were added to yeast cultures in doses ranging from 10^{-7} to 10^{-3} M. Following 4 h incubation, the cultures are assayed for β -galactosidase activity. The values are represented as the rate of β -galactosidase activity divided by the β -galactosidase activity at 10^{-7} 17β -estradiol. The error bars except for BPA, BPF, BPACF and BPS were too small and therefore are not shown on the figure.

Fig. 3 a

Fig. 3 b

Fig. 3 c

Fig. 3 d

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

Fig. 4 a

Fig. 4 b

Fig. 4 c

Fig. 4 d

Fig. 4 Dose-response curves of chemicals. Chemicals were added to MCF-7 cells stock in doses ranging from 10⁻⁷ to 10⁻³ M. Following 144 hr incubation, the cultures were assayed for neutral red. DMSO as served the control. The absorbence was measured with a microplate reader equipped with a 540 nm filter. The readings were averaged and the results expressed as fold increase over control.

at concentrations of 10^{-4} and 10^{-3} M, and significantly increased the percent inhibition in comparison with BPACF and BPS at 10^{-3} M ($p < 0.05$). BPACF increased the percent inhibition and showed the highest percent inhibition at concentrations of 10^{-4} ($71 \pm 2.8\%$) ($p < 0.01$). BPS increased the percent inhibition at concentrations of 10^{-3} M. The remaining 24 chemicals did not show similar effects at the concentrations tested.

The effects of the test chemicals on the proliferation of MCF-7 cells are shown in Figs. 4 a-d. BPA, BPACF and Bis-DMA significantly increased MCF-7 cell growth at concentrations of 10^{-7} , 10^{-6} and 10^{-5} M, compared with the control ($p < 0.01$). BPF and BPS significantly increased MCF-7 cell growth at concentrations of 10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} M, compared to the control ($p < 0.01$). BPA, BPACF and Bis-DMA were cytotoxic at 10^{-4} M and above, while BPF and BPS were cytotoxic between 10^{-4} and 10^{-3} M. The remaining 23 chemicals did not increase MCF-7 cell growth at the concentrations tested, while Bis-GMA, Bis-MEPP, Bis-MPEPP, UDMA, HQ, CQ, P-BPA, E-BPA, BPAEDA and BADGE were cytotoxic at 10^{-4} M and above.

DISCUSSION

The estrogenic activities of chemicals for dental use and BPA-related chemicals were tested by three *in vitro* bioassays. BPA, BPF and BPACF induced β -galactosidase activity by the yeast two-hybrid system, and BPA, BPF, BPACF, and BPS increased the percent inhibition by the fluorescence polarization system, while the remaining chemicals did not show such activity at the concentrations tested. In addition to BPA-related chemicals that were estrogenic in the above two assays, Bis-DMA also significantly increased MCF-7 cell growth in the E-screen test, but the remaining chemicals did not. In this study, a β -galactosidase activity above 0.1, more than 50 percent inhibition and a 1.5-fold increase in cell growth over the control were considered estrogenic activity, respectively. The yeast two-hybrid system appears to be less sensitive than the other assays.

BPA, BPF and BPACF were estrogenic in the yeast two-hybrid system. 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 obtained⁷⁾. However, the conventional yeast assay, the yeast estrogen screen (YES) is cost-effective and sensitive, but requires special processing to detect estrogenic activity¹¹⁾. However, Nishikawa *et al.*⁷⁾ reported that transcriptional activation of ER in YES might occur by spurious interaction with unrelated factors in yeast, resulting in reduced reliability. Coldham *et al.*¹²⁾ reported that BPA showed a dose-related estrogenic effect in YES at 10^{-7} M. Thus, based on the present findings, the yeast two-hybrid system appears to be less sensitive than the YES system. This may be due to the short-term incubation of chemicals with recombinant yeasts (4 hr vs. overnight). β -galactosidase activity can increase with overnight incubation. However, the longer exposure to chemicals may affect the growth and viability of yeast. The cytotoxicity of the

chemicals can be evaluated after 4 hr incubation with yeast. Despite of non-cytotoxicity of BPACF at the concentrations tested, BPACF did not induce β -galactosidase activity at concentrations of 10^{-4} M and above, which suggests it could be specifically estrogenic around 10^{-4} M.

In the present study, we adopted a new 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 ER. Furthermore, this assay can be run at physiological temperatures, requires less than half a day to complete, and involves no radioactivity⁸⁾. BPA, BPF, BPACF and BPS showed estrogenic activity by the fluorescence polarization system, but the remaining chemicals did not. The EC50 values for each chemical were calculated by non-linear least-squares regression and the relative binding affinities (RBAs) for each chemical in relation to that of the natural ligand E2 were also calculated. This RBA value for BPA is similar to those reported by Kuiper *et al.*¹³⁾ and Perez *et al.*¹⁾ in an estrogen/ER competition binding assay using [¹²⁵I]-estradiol and [³H]-estradiol, but is significantly lower than that observed by Waller *et al.*¹⁴⁾ using the CoMFA/3D-QSAR model. BPACF showed the highest percent inhibition at 10^{-4} M, as in the yeast two-hybrid system. This is suggested to be due to the same reason described above.

We also used the E-screen test described by Villalobos *et al.*⁹⁾ This assay compares the cell yield between cultures of breast tumor-derived MCF-7 cells treated with estradiol and cultures treated with different chemicals. BPA, BPF, BPACF, Bis-DMA and BPS showed estrogenic activity by MCF-7 cell growth in the E-screen test, but the remaining chemicals did not. We compared the estrogenic activities of these chemicals to those reported by Perez *et al.*¹⁾ BPA, BPF and Bis-DMA significantly increased MCF-7 cell growth at 10^{-6} M, as they reported. However, in the present study, these chemicals increased proliferation approximately three-fold relative to the control, which is about half that reported by Perez *et al.*¹⁾ Furthermore, they reported that BPAEDA and BADGE showed estrogenic activity at higher concentrations. This may reflect the ability of MCF-7 cells to proliferate in the presence of estrogens. The BUS cell stocks used by Perez *et al.*¹⁾ might be more sensitive than the ATCC cell stocks used in the present study, and from other sources could lead to different results. Bis-DMA significantly increased MCF-7 cell growth at 10^{-7} M over the control, but Bis-GMA, Bis-MEPP, Bis-MPEPP did not. Matsuzawa *et al.*¹⁵⁾ reported that Bis-GMA and TEGDMA did not have estrogenic activity in the E-screen test of MCF-7 cells. However, Nathanson *et al.*¹⁶⁾ reported in an *in vitro* study that Bis-GMA significantly increased MCF-7 cell growth at 10^{-8} M, and suggested that it had an estrogenic effect.

There were occasional discrepancies between the assays. For example, Bis-DMA did not show any estrogenic activity, and BPA, BPF, BPACF and BPS did not show estrogenic activity at concentrations of 10^{-7} , 10^{-6} and 10^{-5} M using the yeast two-hybrid system and fluorescence polarization system, while they did show such activity in the E-screen test. The yeast two-hybrid system and fluorescence polarization system are used to examine the interaction between ER- α and coactivators, and estro-

gen/ER- α competition binding, respectively. Thus, only ER- α binding is required to evaluate estrogenic activity. The cloning of a second estrogen receptor, ER- β , has recently been reported¹⁷⁾, it is also present, together with ER- α , in some human breast cancer cell lines¹⁸⁾. The proliferation of MCF-7 cells in response to endocrine disrupting chemicals could be based not only on ER- α but also on the ER subtype. Another possible factor in this discrepancy, as with Bis-DMA noted above, is that cellular enzymatic systems may play a role and enhance estrogenic activity. Interestingly, Bis-DMA showed estrogenic activity in the E-screen test, but not using the yeast two-hybrid system or fluorescence polarization system. Soderholm *et al.*¹⁹⁾ pointed out, under clinical conditions, that esterase present in saliva could break ester linkages. If the ester linkage of Bis-DMA is attacked by esterase, BPA might be released. It is known that esterase or hydrolase activities are in mammalian serum²⁰⁾, as a result, Bis-DMA might be metabolized to estrogenic metabolites.

Despite the usefulness of these methods for detecting estrogenicity, they also have limitations. For example, in the two-hybrid system, the yeast cells are more robust than mammalian cells and have a cell wall. Some chemicals may not be able to permeate through such a cell wall, which would complicate any interpretation of the results. In the fluorescence polarization system, a decrease in the percent inhibition above 20% was observed for Bis-GMA, Bis-MPEPP, Bis-DMA, 4-META, 4-AETA, CQ and BPAEDA at concentrations higher than 10^{-4} M. Although the reasons for this result are not clear, precipitation and coloring of the screening buffer occurred at this concentration. This phenomenon, or staining by chemicals, might interfere with the system. Bis-GMA, Bis-MEPP, Bis-MPEPP, Bis-DMA, UDMA, HQ, CQ, P-BPA, E-BPA, BPAEDA and BADGE were cytotoxic at concentrations ranging from 10^{-5} to 10^{-3} M. When a chemical was cytotoxic to MCF-7 cells, the E-screen could not be used.

Previous studies have shown that modern dental materials contain many different monomers and additives. MMA, HEMA, Phenyl-P, 4-META, 4-MET, 4-AETA 4-AET, TMPTMA, TMPTA, HQ, CQ, 1G, 2G, 3G, UDMA, NPG, Bis-GMA, Bis-DMA, Bis-MEPP and Bis-MPEPP have been widely used as major oligomers, monomers, photo-absorbers, inhibitors and diluent oligomers in various resin composites and sealants²¹⁻²⁴⁾. Several studies have shown that resin composite fillings might cause pulp inflammation²⁵⁻³¹⁾. Stanley *et al.*²⁵⁾ reported that composites could be considered toxic. Baume *et al.*²⁶⁾ stated that this pulp reaction to composites was mild if there was 1 mm or more remaining dentin. Extracts of resin composites, dentin bonding agents and various composite ingredients have been shown to be cytotoxic, genotoxic and mutagenic in several *in vitro* studies. Yoshii²⁷⁾ examined the relationship between the structure and cytotoxicity of monomers used in dental resin materials, and reported that monomers could be ranked in terms of cytotoxicity as Bis-GMA > UDMA > NPG > 1G > 2G > 3G > 4META > HEMA > MMA. Furthermore, Geurtsen *et al.*²⁸⁾ examined the cytotoxic effects (ED50 concentrations) of 35 monomers or additives in commercial dental resin composites. Among co-monomers, initiators and co-initiators, Bis-GMA, UDMA, Bis-DMA, DMBZ and DMDTA had severe cytotoxic effects, while HEMA, BEMA, CQ, DMPT and DMAPE had moderate cytotoxic

effects. In addition, Schweikl *et al.*²⁹⁾ reported that MMA, HEMA and glutaraldehyde may promote mutagenicity, and suggested that glutaraldehyde and glutaraldehyde-containing bonding agents were clearly mutagenic. In a related study, a dental assistant developed allergic contact dermatitis caused by BPA-containing composites based on epoxy dimethacrylate³⁰⁾. Furthermore, benzoyl peroxide is well known to be a tumor promoter, and is cytotoxic and allergenic³¹⁾. In the studies described above, the side effects of dental materials have been widely investigated. Recently, resin-based dental restorative materials have been targeted as potential sources of xenoestrogenicity, especially BPA, which has been shown to have deleterious side effects³²⁾. However, estrogenic activity has not been considered as a side effect of dental materials. Thus, estrogenicity may need to be considered in future studies of the biological safety of dental materials, once an effective screening method is established.

Since Olea *et al.*³²⁾ reported the possibility that BPA may be released from dental sealants, many studies have examined this release under various conditions and tried to detect BPA released from commercial resin-based restorative materials. While Lewis *et al.*³³⁾ detected the presence of BPA and Bis-DMA in commercial resin-based restorative materials, the levels of these compounds were much lower than those reported by Olea *et al.*³²⁾ Indeed, several studies have reported that BPA is not released from commercial resin-based restorative materials. The use of an aqueous solution for studying BPA release resulted in no release of BPA, since BPA does not dissolve well due to its hydrophobic nature³⁴⁾. However, the selection of an aqueous solution is reasonable clinically, and any bioassay of estrogenic activity should consider the oral environment. We tested the estrogenic activity of extracts from Bis-GMA-based sealants under four conditions (heptanes, 50% ethyl alcohol, 4% acetic acid and artificial saliva). In all of the extracts, BPA was not detected by HPLC and showed no estrogenic activity by the two-hybrid system³⁵⁾. However, BPA appears to be more estrogenic in animal studies than predicted by *in vitro* assays. BPA was examined for its effect on accessory reproductive organs and daily sperm production in male offspring of mice fed this chemical during pregnancy³⁶⁾. From days 11-17 of gestation, female mice were fed an average of 2 ng/g body weight (2 ppb) of BPA (dissolved in oil), and this 2 ng/g dose of BPA permanently increased the size of the preputial glands, but reduced the size of the epididymides³⁷⁾. More recently, Mariotti *et al.*³⁸⁾ reported the estrogenicity of Bis-GMA *in vivo* using the uterotrophic assay. The normalized wet weights of uteri from ovariectomized mice receiving a high dose (100 µg/kg) of Bis-GMA or estradiol were significantly greater (29% and 786% greater, respectively) than those of the ovariectomized control.

Little is known about whether dental materials or BPA itself accumulate in the human body or how they are metabolized. The metabolic fate of an orally administered dose of [¹⁴C]-BPA has been studied in rats. Over an 8-day period, 28% and 56% of [¹⁴C]-BPA was excreted in the urine and feces, respectively, and no [¹⁴C]-BPA residues could be detected in the carcass after 8 days³⁹⁾. Clime *et al.*^{40, 41)} orally administered [¹⁴C]-BADGE to mice and found that it was rapidly excreted; 80% and 11% of the administered [¹⁴C]-BADGE was eliminated in the feces and the urine,

respectively, 0-3 days after a single oral dose. These findings suggest that a chemical like BADGE is not easily absorbed from the gastrointestinal tract. Hikage *et al.*⁴²⁾ demonstrated that the cytotoxicity of various dental resin monomers decreased in the presence of a rat liver S9 mix containing cytochrome P 450 enzymes. The systemic behavior of dental materials and other BPA-related chemicals remains unclear and needs further investigation.

Thus, in the early stage of material research and development, it is important to clarify the estrogenic activity and structure/xenoestrogenicity relationship of chemicals intended for dental use. Estrogenic activity was initially assessed by the subcutaneous administration of chemicals to ovariectomized rats that were then observed for the onset of estrus⁴³⁾. It soon became apparent that diphenyl and diphenyl methane derivatives and stilbene derivatives containing two hydroxyl groups in the *para* positions were estrogenic⁴⁴⁻⁴⁸⁾. Among the BPA related chemicals tested in this study, BPA, BPF, BPACF, Bis-DMA and BPS showed estrogenic activity. Perez *et al.*¹⁾ reported that diphenylalkanes, which have two terminal hydroxyl groups, were assayed to determine the nature of the *para* position: either free (BPA and BPF) or ether or ester bonds. Ester derivatives of diphenylalkanes (BPACF and Bis-DMA) were estrogenic, in contrast to some with ether substituents at the terminal -OH, such as (Bis-GMA, P-BPA and E-BPA), which showed no estrogenic activity in the E-screen test. Moreover, BPS showed weak estrogenic activity although its chemical structure is not similar to those of the diphenylalkanes. BPS consists of two phenolic rings with two oxidant chains joined through a bridging sulfur. A chemical structure carry two terminal hydroxyl groups in the *para*-position could be estrogenic. MMA, HEMA, 1G, 2G, TEGDMA, UDMA, NPG, CQ, TMPTMA and TMPTA did not show estrogen activity in the present study. Estrogenicity may be related to chemical structures bearing benzene rings, since chemicals without benzene rings did not show estrogenic activity⁴⁹⁾. The first studies of active compounds containing only one benzene ring, such as *para*-hydroxy propenyl benzene and some alkylphenolic compounds, have been reported^{50, 51)}. Some phthalate esters containing only one benzene ring have also been shown to exhibit extremely weak estrogenicity⁵²⁾. Phenyl-P, 4-META, 4-MET, 4-AETA, 4-AET and HQ, which have only one benzene ring, but not the structure described above, did not show estrogenic activity.

In conclusion, the present findings show that BPA and some BPA-related chemicals (Bisphenol-F, Bisphenol-A-bischloroformate, Bisphenol-S) were estrogenic by three *in vitro* bioassays, while no related chemicals, except Bisphenol-A-dimethacrylate, were estrogenic *in vitro*. Bis-DMA based dental materials are reportedly no longer manufactured⁵³⁾. Of the chemicals tested in the present study, the diphenylalkanes that carry two terminal hydroxyl groups in the *para*-position, either free (BPA and BPF) or as ester derivatives (BPACF and Bis-DMA), and those with two phenolic rings with two oxidant chains joined through a bridging sulfur (BPS), were estrogenic. However, it is not sufficient merely to determine that they are estrogenic. Additional information, together with *in vivo* and epidemiological examinations, are required. Furthermore, the accumulation of information on various aspects of

materials science and estrogenicity could lead to the development of new chemicals and a possible reconsideration of chemicals currently used in dental applications.

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