In vitro estrogenicity of pit and fissure sealants

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ABSTRACT

Recently dental resinous materials have been targeted as potential sources of xenoestrogens, which mimic the natural steroid hormones and can cause many biologic effects. The purpose of this investigation was to assess the estrogenic action of various types of sealants.

Three filled light cured sealants were included in this study. Each sealant was applied and photopolymerized in eight polyethylene moulds according to the manufacturer's instructions. All specimens were immersed in normal saline for 1 week at 37° C. Samples of eluents at concentrations 5% and 10% v/v were tested for estrogenicity by measuring their effect on the proliferation of the estrogen responsive MCF-7 breast cancer cells. In addition an estrogen insensitive cell line was used as control (MDA-MB-231), in order to exclude a possible cytostatic effect of the tested materials. All assays were repeated 4 times and the results were averaged. The results were analyzed with a 2 way ANOVA and further differences were investigated with Tukey's multiple comparison test at the 0,05 level.

Eluents of the sealants tested at concentrations 5% and 10% v/v did not possess estrogenicity, except of the eluent of one sealant at concentration 10%, which caused an induction of the proliferation rate of the MCF-7 cells. The potential causes of this effect and the clinical implications are discussed.

INTRODUCTION

Estrogenicity is the ability of a group of chemicals called xenoestrogens to exert a biological reaction comparable to that of estrogens.[1] The mechanism proposed for xenoestrogens action is based, in most cases, on their binding to classic estrogenic receptors ERs (ERα and ERβ), at sub-toxic concentrations, inducing estrogenic signals that modify gene expression.[2]

One of these substances, bisphenol-A (BPA), which has a great similarity in structure with 17β-estradiol, is used as a plasticizer in the manufacture of many food covering products.[1] Polycarbonate products subjected to autoclaving or heat sterilization have been found to release BPA.[3] Moreover, for a given exposure period and BPA quantity, the accumulation of BPA in the body may vary as a function of the developmental stage and the gender of the subject.[4]

The biologic effects of BPA on experimental animals have been shown to have mainly endocrine disruptive activities, the most common of which are promotion of cell proliferation in breast cancer cells, prostate cancer in males, early puberty in females and feminization in males. [5] Other biologic effects include neurobehavioral problems, such as autism and attention deficit hyperactivity disorder;[6] development of hyperglycaemia and insulin tolerance;[7] alteration of differentiation of fibroblast cells into adipocytes and altered glucose transport in adipocytes;[8] and elevation of oxidative stress mediators.[9] Moreover BPA has been shown to be a thyroid hormone receptor antagonist that disrupts the THR- mediated transcription. [10]

Estrogen-mimicking compounds, like BPA may also be found in dental materials. Since Bis-GMA which is synthesized from BPA is incorporated in many resinous materials,

it is possible that a small quantity of BPA may remain as an impurity in Bis-GMA-based resins. Moreover, dimetacrylate based restorative materials may contain BPA as a degradation product.[11] Unspecific esterase and other enzymes in saliva attack the dimethacrylate resin matrix leading to a slow and persistent degradation of the material.

The implication of BPA release from dental biomaterials was first reported in a study, which assessed salivary BPA levels in patients with dental sealants. [12] Olea et al confirmed the estrogenicity of the sealants used by proliferation tests of human breast cancer cells. The results of this study caused public concern and, until now, there has been a controversy regarding the actual release of BPA from sealants and their possible estrogenic action. *In vitro* studies failed to detect BPA eluted from properly polymerized sealants.[13, 14] Another paper examining blood samples from 40 dentists, 30 of whom had received sealants, showed that BPA was not found in any of the blood samples from either group, suggesting that if BPA is leached from dental sealants it is not detectable in blood tests.[15] In addition to this, Eliades et al assessed BPA release from orthodontic adhesives and no BPA eluted from the tested materials was found.[16]

A study by Joskow et al examined BPA levels in urine and saliva of adults treated with two different types of sealants. They noted that saliva levels detected in their study were ~1000 times lower than those reported by Olea et al. However they concluded that dental sealants may be a point source for low-level BPA exposure at levels that show health effects in rodents and that further research is required. [17]

Due to the fact that a large number of in vitro studies show that effects of BPA are mediated by mechanisms, with disruption of cell function occurring at doses as low as 1 pM or 0,23 ppt,[6] there is an interest in the evaluation of the biologic action of BPA. Even if a

precise and reliable quantitative estimation is attained, there is still a large window of uncertainty on its potential estrogenicity.

In addition to the foregoing factors, it is important to note that responses to estrogens vary with the saturation of receptor occupancy, and therefore the kinetics of estrogen response deviates from linearity and is described as non monotonic, or inverted U-shaped dose-response relationship.[18] Moreover, there are about 20 forms of bisphenols that share estrogenic action with BPA, like bisphenol-A dimethacrylate (Bis-DMA).[19] Therefore the chosen method for this study was the direct assessment of the estrogenic action of sealants, rather than the presumptuous study of BPA release from materials..

The purpose of this investigation was to assess the estrogenic action of various types of sealants. The null hypothesis of this study was that sealants possess no estrogenic action.

MATERIALS AND METHODS

Specimen preparation

Three light-cured sealants were included in the study (Table 1). Polyethylene moulds (10 mm in diameter and 2 mm in height) were placed on microscopic glass slides covered with transparent cellulose tape. The molds were filled with the sealants according to the manufacturers' instructions, covered with another set of cellulose tape and glass and photopolymerized for 40 s using a light-cured unit (Elipar Visio II, Espe GmbH, Seefeld, Germany) emitting continuous light intensity of 650 mW/cm^2 at 468 nm, as measured with a curing radiometer (Model 100, Demetron Corp., Danbury, CT, USA). All the sealants specimens (n=8 per material) were prepared by the same operator. The specimens were immersed in sterile tubes containing 40 ml of 0.9% w/v normal saline and maintained at 37^{0}C

temperature. During the immersion period the solution was under continuous agitation on a rocking wheel.

Cell Proliferation Assay

MCF-7 and MDA-MB-231 human breast adenocarcinoma cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% foetal calf serum (FCS) (Biochrom KG, Berlin, Germany), at 37^0C, in 5% CO_2, in a humidified incubator. The cells were regularly subcultured by using a 0.25% trypsin-0.3% sodium citrate solution.[20] To evaluate the estrogenicity of the tested materials, the cells were plated in 96-well flat-bottomed microwells (5,000 cells/well) in DMEM/10% FCS. Twenty four h later, the medium was changed to a phenol-free DMEM supplemented with 1% dextran-coated-charcoal-pretreated FCS. After another 24 h new medium was added along with the solutions to be tested. Estradiol, BPA and physiological saline (5% and 10% v/v) were used as positive (estradiol, BPA) and negative (saline) controls. Following an incubation of 5 days, the cells were subjected to an MTT assay: the medium was removed and the cells were incubated with 1 mg/ml MTT [3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide] in serum-free, phenol-red-free DMEM for 4 h. The MTT-formazan produced was solubilized in isopropanol and absorbance at 550 nm (reference 690 nm) was measured. All assays were performed in quadruplicate and the results were averaged.

The statistical analysis of data was performed with a 2-way ANOVA with composition of sealants and concentration of eluent, serving as discriminating variables. Differences were further investigated with Tukey's multiple comparison test at the 0.05 level of significance.

RESULTS

As shown in Table 2, the eluents of the tested materials at concentration 5% did not stimulate the proliferation of the estrogen responsive cell line MCF-7. Even though the stimulatory effect of the eluent of Delton opaque differs statistically significantly from the eluents of the others materials, it is not different from the control.

On the other hand, in the estrogen insensitive cell line MDA-MB-231, BPA at concentration 10^{-8} did not have any stimulatory effect. This cell line, which was used as control to exclude the possibility that effects irrelevant to estrogenicity would interfere with proliferation, did not show any variation in the experimental groups, thus verifying the lack of effect on the estrogen-sensitive cell line.

Table 3 illustrates the results of the estrogenicity assay at concentrations 10% of the eluents. The eluent of Delton opaque differs significantly from the control and the other materials, leading to the conclusion that it possesses estrogenic action. Potential decrease in the vitality of MDA-MB-231 cell line would have implied that the absence of effects on MCF-7 were not true due to lack of estrogenicity but are assigned to a decreasing effect of estrogen action by other material components, as in the case of cytotoxicity reducing the BPA-induced increase in proliferation.

DISCUSSION

Sealants have a greater potential of estrogenicity compared to the other resin materials due to the fact that they contain less percentage of fillers and thus a larger proportion of resin matrix. In addition, the decreased thickness of sealants in relation to their

volume leads to an extremely high surface-to-volume ratio, which results in a substantial portion of the material to be exposed in the oral environment. [21] Moreover, there is a direct exposure of thin films to oral environment. Therefore, priority should be given to the examination of the estrogenicity of these materials, which have been proven to be an effective method of preventing pit and fissure caries in children and adolescents. [22]

The classic method for measuring estrogenic action is the increase of mitotic indices of rodent epithelia.[23] This approach, however, may have limited relevance to humans because the rat hepatic microsomes have been found more effective in reducing estrogenicity compared to human liver.[24] Consequently, assessment of the estrogenicity of substances using the immature rat uterotrophic assay underestimates the potency of BPA in humans. As an alternative, the E-screen assay (a cell proliferation assay) which utilizes a cell line (MCF-7) derived from human breast cancer tissue has been proposed by Soto et al.[25] This cell line shows intense proliferation upon exposure to minute levels of estrogens and is therefore preferred for its sensitivity.

Olea et al were the first that reported the estrogenicity of BPA and its dimethacrylate ester (BPA-DM), which were released from sealants *in vitro*, using the estrogen responsive MCF-7 breast cancer cell line. Schafer et al [26] confirmed the estrogenicity of BPA and Bis-DMA using breast cancer cells (MCF-7, T-47D, ZR75-1). They concluded that the proliferative response of cells to this compound was depended upon the ER content of that cell type. Further support to this notion was provided by Lewis et al, who reported that the type and extent of growth response was highly cell line-dependent.[27]

A previous study in a recombinant yeast cell assay, revealed significantly increased estrogenic activity was found in saliva samples collected immediately after placement of

Delton light cured sealant. [28] However one hour after the placement of the sealant no estrogenicity was detected. Also, Tarumi et al [19] tested the estrogenic activities, using reporter gene assay, of 3 fissure sealants and 5 adhesive resins, which were all unpolymerized. They found that two sealants (Delton, Defender) at concentration 5 μg/mL and greater, possessed estrogenic activity even though they did not contain BPA. However Bis-DMA was found to be included in these sealants, suggesting its involvement in the estrogenicity observed.

It is interesting to note that besides BPA other constituents of the sealants may have estrogenic activity, like a photostabilizer (2-hydroxy-4-methoxy-benzophenone, HMBP), a photoinitiator (2,2dimethoxy-2-phenyl-acetophenone, DMPA) and an inhibitor (2,6-di-tert-butyl-p-cresol, BHT). [29] The minimum concentration of HMBP and DMPA required for estrogenicity was determined to be 1 μmol/L, and both chemicals had estrogenic activity approximately 10 times weaker than BPA.

In the present study the selection of an estrogen insensitive cell line MDA-MB-231 to serve as sham control, led to a more accurate estimation of the estrogenicity of the tested materials. This was due to the fact that the possibility of interaction between the proliferative effect and the cytostatic action of the eluents was excluded.

The eluents of all sealants tested at concentration 5% did not have any stimulatory effect on the MCF-7 cell line. However, at concentration 10% the eluent of one sealant (Delton opaque) showed estrogenic activity. A possible explanation of this finding might be the strong attenuation of the activating light by the opaque tinting agent (TiO$_2$), of Delton Opaque, that leads to increased light back-scattering and therefore incomplete in-depth polymerization. It is known that, polymerization in the deep layers of visible light cured

composites, is significantly affected by the optical properties of the material.[30] Thus, more unpolymerized remaining monomer and unconverted carbon-carbon double bonds (C=C) of the methacrylate moieties immobilized in the network were left in the material and caused this estrogenic action. The unconverted C=C bonds are active sites in the resinous matrix, prone to oxidative or hydrolytic degradation of the sealant. The fact that Delton Clear having the same monomer composition with Delton Opaque but without tinting agents demonstrated no estrogenic action, supports the concept of incomplete in-depth polymerization.

It may be worth noting that *in vitro* studies underestimate the potential estrogenic action of the sealants, because they do not take into consideration the ageing of the material in the intraoral environment, which involves factors such as mastication forces, pH fluctuations, alcohol induced plasticization, microbial and enzymatic activity.[31] It is interesting to note that Schmaltz et al [32] have found that at pH 11, there was a 99, 8% conversion of Bis-DMA to BPA and after a 24h incubation of Bis-DMA in esterase the conversion rate was 82.5%.

However, the American Dental Association (ADA, 2008) has developed a policy statement, which is posted on their website, *"Bisphenol A and dental sealants, composite dental fillings"*. This policy notes that there is evidence that some dental sealants, and to a lesser extent dental composites, may contribute to low-level BPA exposure. However it is noted that this exposure is an acute and infrequent event with little relevance to estimating general population exposures and strongly supports additional research into human exposure to BPA and any health effects that it may cause.[33]

The clinical implication of this study is that the proper polymerization of sealants probably increases the safety of the material. In the present study, sealant polymerization was

performed in closed cells excluding the formation of an oxygen inhibited layer, which is not the case in clinical application. Elimination of the O_2 inhibitor layer of the sealants during their clinical application prevents release of unpolymerized material to the mouth and a possible estrogenic action. This can be done by washing the sealed tooth with an air-water syringe while suctioning fluids and debris from the child's mouth.[1,34] It is also suggested that Bis-GMA based sealants should be replaced by other materials possessing high molecular weight monomers without aromatic rings.

CONCLUSIONS

- The eluents of the sealants tested at concentration 5% did not have any estrogenic activity

- The eluents of the sealants tested at concentration 10% did not possess any estrogenic activity, except the eluent of one sealant (Delton Opaque).

What this paper adds

- In this study the selection of an estrogen insensitive cell line MDA-MB-231 to serve as sham control, led to a more accurate estimation of the estrogenicity of the tested materials. The possibility of interaction between the proliferative effect and the cytostatic action of the eluents was excluded.

- The strong attenuation of the activating light by the opaque tinting agent (TiO_2), of one tested material led to increased light back-scattering and therefore incomplete in-

depth polymerization. This might be the explanation for the estrogenic activity found for this material.

Why this paper is important to paediatric dentists

- The incomplete polymerization of sealants (strong attenuation or scattering of the activating light, formation of an oxygen inhibited layer) may be hypothesized to lead to the release of substances that might have estrogenic activity.

- Although the use of sealants is basically safe, the proper and complete polymerization increases the safety of the material.

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Table 1: The sealants tested		
Material/ Lot	**Composition***	**Manufacturer**
Delton Opaque (080428)	Bis-GMA, Bis-DMA, TEGDMA, CQ, amine, Benzyl, fumed SiO_2, TiO_2	Dentsply International York , PA, USA
Delton Clear (080408)	Bis-GMA, Bis-DMA, TEGDMA, CQ, amine, Benzyl	Dentsply International York , PA, USA
Ultraseal XT Plus Clear (B3QJ2)	Bis-GMA, DUDMA, TEGDMA, CQ, amine, SiO_2	Ultradent Products, S. Jordan, UT, USA

***According to manufacturers' files. Bis-GMA:Bisphenol glycidyl dimethacrylate, Bis-DMA: Bisphenol dimethacrylate, TEGDMA: Triethyleneglycol dimethacrylate, DUDMA: Diurethane dimethacrylate, CQ: Camplorquinone**

Table 2:	Stimulatory effects of eluents at concentration 5% on the MCF-7 proliferation	
Material	**Mean (SD)**	**TUKEY GROUPING***
Delton opaque (DO)	112,64 (21.36)	A
Control (C)	101,61 (10,83)	A B
Delton clear (DC)	91,53 (9,73)	B
Ultraseal XT (U)	90,67 (12,58)	B

***Values with at least one same letter do not differ statistically significantly at the level of 0.05*

Table 3: Stimulatory effects of eluents at concentration 10% on the MCF-7 proliferation		
Material	**Mean (SD)**	**TUKEY GROUPING***
Delton opaque (DO)	169,65 (51,52)	A
Delton clear (DC)	122,10 (35,67)	B
Ultraseal XT (U)	117,31 (26,02)	B
Control (C)	100,03 (8,61)	B

***Values with at least one same letter do not differ statistically significantly at the level of 0.05*