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Oestrogenicity of orthodontic adhesive resins

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SUMMARY The purpose of this study was to assess the oestrogenic action of a chemically cured, no-mix (Rely-a-Bond) and a light-cured (Reliance) orthodontic adhesive resin. The adhesives were bonded to 40 stainless steel maxillary incisor brackets (Diamond) divided into two equal groups, employing a method which simulated the clinical handling of materials. In total, three series of specimens were prepared for each adhesive–bracket group. All specimens were immersed in normal saline. Samples of eluent were removed from each group at 1 day and 1 week following incubation and tested for oestrogenicity by measuring their effect on the proliferation of the oestrogen-responsive MCF-7 breast cancer cells, while an oestrogen-insensitive cell line (MB-231 human breast adenocarcinoma) was used as a control.

Three-way analysis of variance with adhesive, concentration of eluent, and immersion period were used as discriminating variables. No evidence was found of stimulation of proliferation of these cells, indicating the absence of any oestrogenicity of orthodontic adhesive eluents.

Introduction

Oestrogenicity is a relatively recently described property of certain polymeric molecules such as bisphenol-A (BPA), to express biological effects similar to those induced by natural oestrogens (Directorate of General Health and Consumer Protection, European Commission, 2003). Although originally discovered some decades ago (Colborn *et al.*, 1993), the concerns arising from the action of these polymers reached an alarming level during the past few years because of the publication of a substantial body of research reporting biological effects associated with these compounds. These effects basically include a wide array of biological phenomena affecting many tissues and systems (Welshons *et al.*, 2003; Munoz-de-Toro *et al.*, 2005; Wozniak *et al.*, 2005).

Recent studies have demonstrated the cohort of phenomena accompanying the exposure of organisms to BPA, which include hormonal-related effects such as early puberty in females and feminization in males; higher risk for breast cancer in females and prostate cancer in males (Timms *et al.*, 2005); induction of calcium influx, which leads to prolactin release and associated behavioural effects (Palanza *et al.*, 2002); development of hyperglycaemia and insulin tolerance (Alonso-Magdalena *et al.*, 2006); elevation of oxidative stress mediators (Ooe *et al.*, 2005); and upregulation of the cAMP response element-binding factor, which inhibits apoptosis (Quesada *et al.*, 2002).

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. Exposure of infants leads to higher BPA body levels relative to that during adulthood, because of the absence of enzymes capable of metabolizing BPA to its biologically inert form. Also, a sexual dimorphism is implied by research indicating higher plasma BPA levels in male than female foetuses, even after correcting for a positive correlation between body weight and BPA concentration (Schönfelder *et al.*, 2002).

As a general rule, oestrogenic action is confined to molecules possessing a double benzoic ring. In dentistry, such polymers include Bis-GMA and polycarbonate products and, therefore, potential BPA sources in dental materials are confined to sealants, composites, adhesives, and polycarbonate aesthetic brackets. Relevant research has demonstrated inconclusive evidence on the actual release of BPA. Although no release has been reported for most polymeric materials (Nathanson *et al.*, 1997; Schmalz *et al.*, 1999), there are also some publications which show variable release from sealants and polycarbonate brackets (Pulgar *et al.*, 2000; Watanabe *et al.*, 2001; Watanabe, 2004).

The biological action of BPA has been reported to occur at very low concentrations, within the range of the detection threshold of the majority of standard analytical techniques (Welshons *et al.*, 2003). Specifically, phthalates, such as octaphenol, are capable of altering the uptake of dopamine by hypothalamic cells, at levels as low as 10 ppt (parts per trillion or pg/l; Christian and Gilles, 1999). Therefore, even if a precise and reliable quantitative estimation of BPA is attained, there is still a large window of uncertainty on its potential oestrogenicity. Moreover, there are about 20 forms of bisphenol, and some of them share oestrogenic action with BPA, such as bisphenol-A dimethacrylate (Tarumi *et al.*, 2000). Therefore, the direct assessment of the oestrogenic action of aged adhesive eluents may be the method of choice for the study of potential oestrogenic action of orthodontic polymers.

The purpose of this investigation was to study the oestrogenic action of orthodontic adhesive resins. The null hypothesis of this study was that Bis-GMA-based orthodontic adhesives have no oestrogenic action.

Materials and methods

Specimen preparation

A chemically cured, no-mix (Rely-a-Bond, Reliance Orthodontic Products, Itasca, Illinois, USA) and a visible light-cured adhesive (Reliance) were selected for the study. Forty stainless steel upper incisor brackets (Diamond, Ormco, Glendora, California, USA) were divided into two equal groups. The first group was bonded with the no-mix adhesive as follows: a standardized volume of adhesive was applied to the bracket base and the bracket was pressed firmly on a surface covered by a cellulose film to facilitate detachment of the bracket–adhesive complex with a recovery of the set material; any excess resin was removed before polymerization. The visible light-cured adhesive specimens were photopolymerized utilizing a light-curing unit (Elipar Visio II, Espe GmbH, Seefeld, Germany) emitting a light intensity of 650 mW/cm^2 at 468 nm, as measured with a curing radiometer (Model 100, Demetron Corp., Danbury, Connecticut, USA). In this group, the brackets were pressed against a yellowish background surface of 75 per cent reflectance. Irradiation was performed from the incisal and cervical bracket edges for 10 seconds at each side. All bonding procedures were performed by the same operator (TE).

Specimen construction yielded two groups (metallic/chemically cured and metallic/light cured) of 20 brackets each, thus representing an average bonded case. The bonded brackets were stored for 5 minutes at 37°C and 50 per cent relative humidity. Each group was then immersed in sterile tubes containing 40 ml of 0.9 per cent w/v normal saline and maintained at a temperature of 37°C. During the immersion period, the solution was under continuous agitation on a rocking wheel. In total, three series of specimens were prepared for each adhesive–bracket group for oestrogenicity assays.

Cell proliferation assay

The assays involved two cell lines: an oestrogen sensitive (MCF-7) and an oestorgen insensitive (MDA-MB-231 human breast adenocarcinoma). The reason for their selection was to exclude the possibility that the decreased proliferation of cells induced by the adhesive eluent found previously (Gioka *et al.*, 2005) would mask a potential induction of proliferation due to the oestrogenic effect.

Cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10 per cent foetal calf serum (FCS; 0 KG, Berlin, Germany), at 37°C in 5 per cent CO_2, in a humidified incubator. The cells were regularly subcultured using a 0.25 per cent trypsin–0.3 per cent sodium citrate solution (Kletsas and Stathakos, 1992). To evaluate the oestrogenicity of the tested materials, the cells were plated in 96-well flat-bottomed microwells (5 000 cells per well) in DMEM/10 per cent FCS. Twenty-four hours later, the medium was changed to a phenol-free DMEM supplemented with 1 per cent dextran-coated charcoal pre-treated FCS. After a further 24 hours, new medium was added along with the solutions to be tested. Oestradiol, BPA, and physiological saline (5 and 10 per cent v/v) were used as positive (oestradiol, BPA) and negative (saline) controls. Following an incubation of 5 days, the cells were subjected to an MTT [3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide] assay: the medium was removed and the cells were incubated with 1 mg/ml MTT in serum-free, phenol red-free DMEM for 4 hours. 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.

Statistical analysis of data was performed with a three-way analysis of variance with adhesive (chemically cured or light-cured), concentration of eluent, and ageing period (1 day and 1 week) 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 Figure 1, both oestradiol and BPA, at the indicated concentration (10^{-7} and 10^{-8} M), were found to stimulate the proliferation of the MCF-7 cells. However, eluents from the chemically and light-cured adhesives and from the two incubation periods (1 day and 1 week) were unable to stimulate cell proliferation. Likewise, normal saline at concentrations of 5 and 10 per cent also had no stimulatory effect on MCF-7 proliferation, and was used as a control for the eluents.

In comparison, in the oestrogen receptor α-negative MDA-MB-231 cell line, oestradiol and BPA did not stimulate any proliferation while the same was observed for all four eluents tested (Figure 2). Collectively, the data from both cell lines indicated that no oestrogenicity was present in the eluents from these resins.

Discussion

The oestrogenicity in eluents of the tested adhesives was measured by an established assay, namely the estimation of the proliferation of the oestrogen-responsive cell line. These cells are known to express oestrogen receptor-α, which is of primary importance for the proliferative effect of oestrogens (Maggiolini *et al.*, 2001). The classic method for measuring oestrogenic action is the increase of mitotic indices of rodent epithelia (Al-Hiyasat *et al.*, 2004); however, this approach

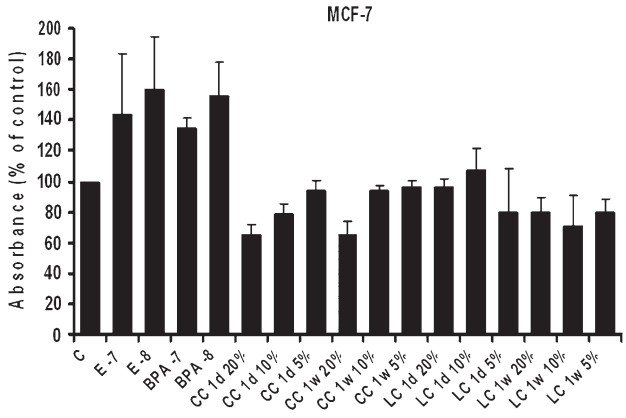

Figure 1 Effect of the chemically cured (CC) and light-cured (LC) adhesive eluents on the proliferation of MCF-7 cells. Positive controls (BPA, oestradiol) demonstrate a proliferative effect, whereas no stimulation is shown by the adhesive eluents and the negative control (normal saline). C, normal saline; E-7, oestradiol at concentration 10^{-7}; E-8, oestradiol at a concentration 10^{-8}; BPA-7, bisphenol-A at a concentration 10^{-7}; BPA-8, bisphenol-A at a concentration 10^{-8}.

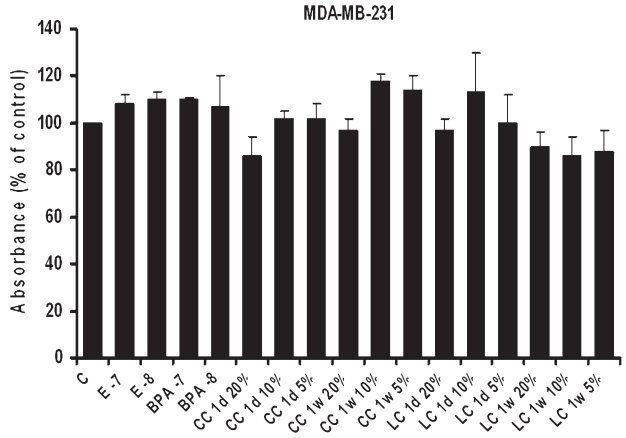

Figure 2 Effect of the chemically cured (CC) and light-cured (LC) adhesive eluents on the proliferation of the oestrogen-insensitive MDA-MB-231 cell line. Both positive (BPA, oestradiol) and negative (normal saline) controls demonstrate no effect on the proliferation of cells. Similarly, no effect is induced by the adhesive eluents. C, normal saline; E-7, oestradiol at a concentration 10^{-7}; E-8, oestradiol at a concentration 10^{-8}; BPA-7, bisphenol-A at a concentration 10^{-7}; BPA-8, bisphenol-A at a concentration 10^{-8}.

may have limited relevance to humans. This is because rat hepatic microsomes have been found to be more effective in reducing oestrogenicity compared with human liver (Elsby *et al.*, 2001). Therefore, assessment of the oestrogenicity of substances using the immature rat uterotrophic assay underestimates the potency of BPA in humans. As an alternative, a cell proliferation assay has been proposed, which utilizes a cell line (MCF-7) derived from human breast cancer tissue (Soto *et al.*, 1986). These cells show intense proliferation upon exposure to minute levels of oestrogens.

The basic differences between the study of common toxicants or other hazardous materials and BPA, relate to the fact that natural hormones such as 17β-oestradiol induce effects at concentrations far below the levels at which all hormone receptors become bound. Once all receptors are occupied, a further increase in natural hormone levels does not result in an increased response. Conventional testing of substances for toxicological impact assessment involves exposure to levels many times higher than those required for complete receptor binding. Thus, the lack of response to excessively high concentrations of effectors may be misinterpreted as a lack of effect (Welshons *et al.*, 2003). On the other hand, the effects of BPA on tissues follow a non-monotonic curve pattern, which is characterized by intense reactivity at low levels and no response at very high ones (vom Saal *et al.*, 1997). Lastly, the concept of 'critical concentration', referring to the required amount of substance to induce effects, may not apply in the case of exposure to BPA. This is due to the inability of the defence system to recognize BPA as a foreign substance owing to its resemblance to a natural hormone, and the sensitivity of target cells and tissues to oestrogens.

The implication of BPA release from dental biomaterials was first reported in a study that assessed salivary BPA levels in patients with dental sealants (Olea *et al.*, 1996). Nonetheless, the clinical application of orthodontic adhesives is vastly different from that of restorative composite resins and sealants. This is due to the decreased thickness of sealants relative to their volume, which leads to an extremely high surface-to-volume ratio. As a result, a substantial portion of the material is exposed to the oral environment, thereby maximizing the effect of mechanical and chemical ageing. The latter includes the effect of cyclic loading arising from masticatory loads, that of microbial and enzymes as well as large pH fluctuations, thus contributing to the formulation of a particularly potent ageing environment. On the contrary, orthodontic adhesive exposure to the oral environment involves only the bracket peripheral margins with an average thickness of 150–250 μm (Eliades *et al.*, 1991), the functional stimuli are minimal, and the subsequent effect of ageing may not be potent.

Previous studies have shown that adhesive extracts may have a mild cytostatic effect (Gioka *et al.*, 2005). Such an effect was observed also in this study when a higher concentration of eluent, in the order of 20 per cent v/v, administered to cells was found (data not shown). Thus, one could hypothesize that the absence of a potential oestrogenic action of the eluent in this study could be the result of an antagonistic pattern of action between the proliferative effect of adhesive eluent, owing to the oestrogenic activity and the inhibitory effect due to the proven cytostatic effect. To assess this hypothesis, an oestrogen-insensitive cell line, MDA-MB-231, was selected to serve as a sham control, and the results excluded the possibility of interaction between these two modes of actions.

Whereas, *in vitro* ageing protocols such as the one employed in this study cannot reliably simulate the intraoral

environmental milieu (Söderholm, 2003), retrieval analyses may not be applied in this case because of the necessity to incorporate the eluent in the media of cell cultures. The latter may not be feasible because of the exposure of materials to the oral cavity of subjects, which is colonized by intraoral flora. To facilitate a large safety window, this study was performed on brackets bonded to free adhesive surfaces, and therefore the entire surface of the adhesive, which corresponds to the surface bonded to the enamel, was exposed to normal saline.

Conclusions

No oestrogenic activity of adhesives was documented in this *in vitro* assay employing a standard model for the assessment of oestrogenicity of materials.

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