Cytotoxicity and estrogenicity of Vivera® retainers

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DDS, Ajman University of Science and Technology, 2008

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ABSTRACT

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Aims:

The aim of the present study is to investigate the cytotoxicity and estrogenicity of Vivera® retainers by assessing their biological behavioral effects: as-received from the manufacturers, and after retrieval from patients.

The null hypothesis of this study is that Vivera® retainers, both as-received or after retrieval from patients, have no cytotoxic or estrogenic effect.

Materials and Methods:

The study sample consisted of six sets of Vivera® retainers, three as-received from the manufacturer and three retrieved from three consecutive patients of the Postgraduate Orthodontic Clinic, Hamdan Bin Mohammed College of Dental Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates. All participants agreed to their inclusion in this research study. With regard to the retrieved retainers, these were retrieved from the patients after four weeks of use. All sets in the study consisted of a maxillary and a mandibular appliances.

The evaluation of the cytotoxicity and estrogenicity of all retainers took place in the Laboratory

of Cell Proliferation and Ageing, Institute of Biosciences and Applications, National Center for Scientific Research "Demokritos", Athens, Greece. The retainers were transferred from Dubai to the laboratory in Athens by one member of the research team in a way that ensured that their physical condition remained unchanged from the time they were removed from the patients or delivered by the company. All retrieved retainers were divided in two equal parts randomly regardless of being upper or lower component. Each one subjected to either mode of sterilization procedures, i.e. gamma-irradiation or autoclaving. The as-received retainers were divided into three equal parts randomly as well. Two parts were sterilized, with each part using one of the above-mentioned procedures, while the third part of as-received retainers was not subjected to any sterilization mode, so as to test the effects of the sterilization procedure

Subsequently, all samples were immersed in sterile normal saline (NaCl 0.9% w/v) with each sample in different container and incubated for fourteen days at 37 °C. A sample of normal saline without any retainer was incubated in the same conditions in parallel with the study samples, to be used as negative control. After sterilization, all retainers, which had been treated following specific allocation and procedures of sterilization, were aliquoted and kept at -20 °C to maintain its integrity until further experimental use. Samples obtained from incubation of as-received / unsterilized retainers were considered to be identical.

The estrogenicity assays involved 2 cell lines, i.e. the estrogen-sensitive MCF-7 and the estrogen-insensitive MDA-MB-231 (both from human breast adenocarcinoma), in order to exclude the possibility that a decreased proliferation of cells induced by the retainer eluent would mask a potential induction of proliferation due to estrogenicity.

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10%

Fetal Bovine Serum, at 37 °C, in 5% carbon dioxide, in a humidified incubator. The cells were regularly subcultured by using trypsin-citrate solution. To evaluate the estrogenicity of the samples, the cells were plated in 48-well flat-bottomed microwells (10,000 cells per well) in DMEM and 10% fetal calf serum. Twenty-four hours later, the medium was changed to phenol-free DMEM supplemented with 2% fetal calf serum pretreated with dextran-coated charcoal, along with the solutions to be tested, at concentrations varying from 5% to 20% vol/vol. β-Estradiol was used as positive control, and normal saline solution was used as negative control. After six days of incubation, with the medium renewed at day three, the cells were detached using trypsin-citrate solution and counted using a Z1 Beckman-Coulter counter. The assays were performed in triplicate and the results averaged. The statistical analysis of data was performed with 2-way analysis of variance (ANOVA) with appliance and concentration as predictors. Differences were further investigated with the Tukey multiple comparison test at a 0.05 level of significance.

Results:

An initial experiment was performed using 3 samples, corresponding to as-received retainers, to assess the effects of the two sterilization procedures and the third served as control. None of the samples, at any concentration tested, induced a proliferation of MCF-7 cells compared to the negative control. This was in contrast to the pronounced stimulation by all three β-estradiol concentrations (within the physiological limits) tested. However, after gamma-irradiation, the retainer appearance appeared altered, having acquired a yellowish color reminiscent of the effect of ultraviolet light on plastic materials. Hence, the sterilization through gamma-irradiation was considered a possible source of damage the plastic, and autoclaving was finally chosen as the preferred mode of sterilization.

Accordingly, 3 samples, corresponding to the retrieved retainers from the three patients were evaluated, in comparison to 2 samples from as-received retainers (either autoclaved or not).

No significant MCF-7 proliferation was induced by the three samples compared either to the eluents from as-received retainers or to the negative control. As expected, β-estradiol induced a potent stimulation of MCF-7 cell proliferation, while no effect was observed on MDA-MB- 231 cells.

Thus, the null hypothesis was accepted meaning that Vivera® retainers, either as-received or after retrieval from patients, possess no cytotoxic or estrogenic effects.

Conclusions:

Based on this study, which was performed with the aim of testing the cytotoxic and estrogenic behavior of both as-received and retrieved Vivera® retainers, there was no significant release of substances with estrogenic activity after incubation in normal saline for two weeks at body temperature.

DEDICATION

To my dearest mother and father, my success in life could not have been without your love, prayers and belief in me.

To my beloved siblings, thank you for your sincere wishes and special thanks for my sister, Engineer Marwa for her generous support.

To all my colleagues in the Department of Orthodontics, thank you for your continuous support.

DECLARATION

I declare that all the content of the thesis is my own work. There is no conflict of interest with any other entity or organization.

Name: Shaima Rashid Al Naqbi

Signature:

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1. Introduction

After orthodontic treatment, the stability of the occlusion achieved is one of the major goals that concern both the orthodontist and the patient under treatment. As post-treatment changes can occur, not only due to the instability of the new occlusion produced by orthodontic therapy but also related to growth, maturation and aging of the dentition in the life processes, it is crucial for appropriate retention protocols to be scheduled for every individual. Among the different types of retainers used for this purpose, those having esthetic advantages, comfort of use, and ease of hygiene maintenance are clearly the most preferable. Clear, removable thermoplastic retainers belong to this category and have, as a consequence, become popular with patients.

Vivera® is a clear retainer that has been recently introduced by the Align Technology Inc. (Santa Clara, California, USA). No claims have been made regarding its material to have any significant differences from their clear aligner counterparts yet, where cytotoxicity and estrogenicity have been tested. Nowadays, concerns about patients' safety receive paramount attention. Since no tests on this version of clear retainers have been published regarding the previously mentioned properties, it was considered useful to perform this kind of assessment.

2. Literature Review

2.1 Retention

2.1.1 Definition

Retention of the teeth in their new position after orthodontic treatment has been defined as "the holding of teeth following orthodontic treatment in the treated position for the period of time necessary for the maintenance of the result" (Moyers, 1973) or as "the holding of teeth in ideal esthetic and functional position" (Riedel, 1969).

2.1.2 Rationale of retention

The long-term stability of the achieved occlusion is a major goal for every orthodontist constituting a major aim at the onset of every treatment and is indeed a vital factor for the satisfaction of every patient undergoing orthodontic therapy as well (Bondemark et al., 2007). It has been, and continues to be a challenge for the orthodontic profession because of a variety of underlying factors contributing to the instability of the occlusion. Those factors are divided into two categories: (a) Growth, maturation, and aging of the dentition throughout life. (b) The inherent instability of the occlusion achieved by orthodontic treatment (Nanda and Zernik, 1993).

As continuing growth can be expected to occur in preadolescent and adolescent patients, some unwanted changes are liable to occur if orthodontic treatment is completely discontinued at this stage. Continuation of excessive mandibular growth in Class III malocclusions and in deep bite cases where the mandible continues to grow in anterior rotation or/and increasing the curve of Spee by time producing further deepening of the bite are two good examples of this phenomenon (Cambpell, 1983; Nielsen, 1993).

Maturation of the dentition throughout life is also a very pertinent factor. It is expressed mainly

in the form of continuous lower incisor crowding in treated orthodontic patients, or patients with normal occlusion left without treatment (Sinclair and Little, 1983; Little et al., 1988; Al Yami et al., 1999). Continuous increase of palatal height up to adulthood resulting in a continuous slow eruption of the teeth is another example of the maturation of the dentition (Thilander, 2009).

Age-related changes in the occlusion during adulthood can be due to ongoing oral degeneration, such as periodontitis, that result mainly in the spacing and flaring of maxillary anterior teeth. In some circumstances, tooth migration occurs towards the edentulous area of the oral cavity (Melsen, 2012). It should be noted that the above-mentioned factors can play a role even in individuals who have never received orthodontic therapy. The tendency of a treated occlusal changes rebound constitutes another reason for orthodontic relapse. Rotated teeth might form the best example: here periodontal fibers can have the forces to rotate the teeth back to their former positions even three months after orthodontic derotation (Reitan, 1959; Beertsen, 1979; Edward, 1988).

Taken together, these factors illustrate the need for thorough and systematic approach to minimize orthodontic post-treatment changes (Melrose and Millett, 1998).

2.1.3 Types of retainers

Retainers can either be fixed or removable. Fixed (bonded) retainers are attached to the palatal and/or lingual surfaces, mainly of anterior teeth, using an acid-etch composite resin. They are commonly one of three types: (a) multistrand wire retainer bonded to each tooth involved in the intended segment, (b) rigid canine-to-canine retainer bonded to the canine teeth only or, (c) reinforced fibers bonded to each tooth involved in the intended segment as well. The fitting of these types of retainers is technique-sensitive; it is crucial that the bonding field is thoroughly clean and dry. In addition, the wire or fiber used should be completely passive.

Removable retainers can be classified into acrylic-based retainers and clear overlay retainers. There are several different types of acrylic-based retainers including the Hawley retainer that is considered to be the classic removable retainer, presenting significant advantages and several modifications in design had been found thereafter. The Jensen, Begg and Van der Linden retainers are other examples of acrylic-based retainers (Van der Linden, 2003; Littlewood, 2013).

Clear overlay (vacuum-formed) retainers are appliances made from thin transparent plastic, and fit closely over the teeth and are almost invisible. They have become popular because of possessing advantages including: superior esthetics, less interference with speech, easy to fabricate, cost effective, less likely to break and superior retention of lower incisors (Sheridan et al., 2003; Rowland et al., 2007; Littlewood, 2013; Atik et al., 2016). Recently Align Technology Inc. (San Jose, CA, USA) introduced a clear overlay device with a similar configuration of aligner marketed as the Vivera® retainer (http://www.invisalign.fr/en/what-is-invisalign/Pages/ViveraRetainers.aspx).

2.2 Clear retainers

2.2.1 History of thermoplastic retainers

The idea goes back to the 1940's when Kesling revolutionized the orthodontic world with the development of an appliance called a "tooth positioner" which was primarily used to reduce the spaces left after debanding. The technique involved taking impressions from a patient who were about finishing or only having mild malocclusion, then stripping the plaster off the resulting models and resetting the teeth into an ideal position. A series of such thermoplastic tooth positioners would be fabricated to be used by the patient to progressively move the teeth to the desired position at the end of active orthodontic treatment (Kesling, 1946).

Somewhat later, the dental contour appliance was invented by Nahoum (1964) characterized by

more or less the same idea of using a clear plastic overlay appliance to move teeth incrementally to ideal positions. A few years after, vacuum-formed retainers were introduced and named the "invisible" retainers by Ponitz (1971). For many years after their introduction, vacuum-formed retainers were still fabricated using the same technique. Casts are poured from the impressions of the patient. A sheet of plastic is heated until the plastic softens enough to be formed and a vacuum unit removes the air between the plastic and the cast so that atmospheric pressure molds the plastic to closely follow the contours of the patient's cast. The Essix retainer, essentially similar to the vacuum-formed retainer of previous years, but utilizing thinner and stronger material, was subsequently presented by Sheridan et el. (1993). Taking advantages of the evolution in 3D digital imaging, clear overlay active appliances were fabricated using computer aided design - computer aided manufacture (CAD/CAM) methods. The Invisalign® technique was initially launched utilizing this concept in 1997 by Align Technology Inc. (San Jose, CA, USA) (Wong, 2002). This concept has been followed by several other companies to produce various similar systems including Orthocaps®, Ecligner®, MTM Dentsply® and Accusmile® have become available on the market. Vivera® retainers are the passive version of Invisalign® aligners that were introduced almost five years ago (http://www.invisalign.fr/en/what-is-invisalign/Pages/ViveraRetainers.aspx).

2.2.2 Introduction of Invisalign®

Increasing interest in adult orthodontics has been accompanied by an increased demand for esthetic alternatives to conventional fixed orthodontic appliances (Meier et al., 2003; Nedwed and Miethke, 2005). This led Mr. Zia Chisti and Ms. Kelsey Wirth, who were entrepreneurs and orthodontic patients on retention, to come up with the idea of using CAD/CAM technology combined with laboratory techniques to fabricate a series of clear plastic aligners to move teeth in 1997. This was marketed as the Invisalign® system in the United States (Wong, 2002). The

devices are made of 0.75mm thick laminated foils composed of polyurethane with methylene diphenyl diisocyanate and 1.6 hexanediol (EX 40). The aligners are fabricated based on the Kesling (1946) principles in which vinyl polysiloxane impressions of the dental arches, including the alveolar processes and bite registration, are taken along with intraoral and extraoral photographs. Miethke (2012) has described in detail the process of manufacturing Invisalign® aligners as follows; *"The impressions are scanned by a computed tomography (CT) scanner while rotating in front of an amorphous silicon X-ray sensor. From the database a set of virtual models are generated. The precision of the scanning process is around 100µm. Eventual artifacts are corrected by a process of tooth shaping based on the operator's knowledge of tooth anatomy and the patient's photographs. The occlusion is established based on the bite registration supplemented by the photographs, and a software program for collision control. In addition, the gingival sulcus is marked and a virtual gingiva is draped over the alveolar processes. This serves to define the margin of the aligners during manufacturing and to allow a more realistic visual presentation… The technician will then rearrange all the teeth according to the treatment plan submitted by the orthodontist and determine the sequence by which the individual teeth should be moved… The movement can be variously programmed between 0.10 and 0.33mm. Depending on the complexity of the malocclusion, the sequence and the velocity of movements, the number of necessary aligners required can reach 70 or even more. After being prepared in the Align laboratory, the virtual model is sent to the clinician, who can assess the treatment with an animation program called 'ClinCheck®' … Once the treatment goal has been accepted, Align Technology will transfer the virtual model into a physical resin model using a stereolithographic method. During stereolithography, a laser cures liquid polymer by photoactivation. The cleaned and refined acrylic models will then be loaded into an automat that heats, pressure forms and laser marks all the raw aligners, which are then transferred to a cutting automat. This machine*

will trim aligners after which they all are separated from the stereolithographic model, polished, disinfected, packaged and finally shipped to the respective doctor".

Each aligner is intended to be worn for nearly twenty two hours per day except for eating, cleaning, brushing, and flossing over a period of about two weeks. They are then changed and replaced by the succeeding one. Each aligner is designed to move a tooth or a small group of teeth about 0.25–0.30 mm (Melkos, 2005).

As mentioned previously, patients are motivated towards a preference for such types of treatment modality for esthetic reasons. A web-based survey conducted in the United States involving participation by 200 lay people concluded that clear trays were better accepted socially during the course of orthodontic therapy than any alternative orthodontic appliances (Ziuchkovski et al., 2008). In addition, this kind of orthodontic device has proven to induce the lowest levels of adverse oral symptoms, including sores on the tongue, cheek, or lip, halitosis, and food accumulation, or even pain experience when compared with buccal or lingual orthodontic appliances (Miller et al., 2007; Shalish et al., 2012; Schaefer and Braumann, 2010). However, other study showed that these devices are reported to cause relatively higher levels of pain during the first days after insertion than other types of appliances (Shalish et al., 2012).

Although the Invisalign® technique has been marketed mainly to orthodontic specialists, over the years it has been utilized by large numbers of other specialists and general practitioners as well (Vice´ns and Russo, 2010).

The Invisalign® techniques present several limitations regarding their biomechanical capabilities and performance on teeth movement (Djeu et al., 2005; Baldwin et al., 2008; Kravitz et al., 2009; Pavoni et al., 2011; Rossini et al., 2015). However, during the last few years specific

improvements in the technique have facilitated more complex types of orthodontic teeth movements (Simon et al., 2014; Li et al., 2015; Weir, 2016).

The structural stability of Invisalign® aligners has been subject to testing by Schuster et al. (2004). There were some significant morphological differences seen in the used aligners in relation to the new ones involving abrasion at the cusp tips, adsorption of integuments at stagnation sites, and localized calcification of the biofilm developed during intraoral use. Similarly, their mechanical properties were adversely affected during intraoral aging (Bradley et al., 2016).

Concerning the leaching of biologically active substances, the same group of Schuster et al. (2004) could not find any traceable amount of substances in an ethanol aging solution after immersion of aligner specimens for two weeks at 23° C. It should be noted, however, that the intraoral environment has a significantly different aging pattern involving abrasive wear arising from mastication. Therefore, the reactivity and biological properties of the appliance material cannot be concluded by this study alone (Schuster et al., 2004). Subsequently, Eliades and co-workers (2009) also failed to find any evidence likely to create biological concerns. Their study failed to find any cytotoxic and estrogenic activity of the device materials when tested *in-vitro*. However, Premaraj et al. (2014) were the first to show that the Invisalign® material produced some negative biological effects on oral epithelial cells in terms of changing their viability, membrane permeability, and adhesion pattern in a saline-solution environment. These findings were under *in-vitro* conditions. However, the authors mentioned that, in the oral environment the presence of saliva might offer a kind of protection.

2.2.3 Vivera® retainers

Vivera® is a type of clear overlay retainer (transparent thermoplastic tray-like appliance) with

separate components fitting over the upper and lower dental arches following orthodontic treatment. It was introduced by Align technology (San Jose, CA, USA) a few years ago and follows the same 3D manufacturing procedure used in the fabrication of Invisalign® aligners. It can be used as the sole retainer or also in conjunction with a lingual bonded retainer. The latter option should be clearly stated when requesting fabrication of this retainer thus avoid confusion while manufacturing.

Since any type of retainer that is esthetic, comfortable and relatively inexpensive tend to become the appliance of choice by most people (Sheridan et al., 1992; Lindauer and Shoff, 1998; Hichenes et al., 2007), Vivera® retainers present obvious advantages.

From an esthetic perspective, the color stability and transparency of the retainer during its use is a critical issue. Polyurethane is the basic constituent polymeric component used in Invisalign® aligners material and is not entirely inert since the material is affected by heat, moisture, and prolonged contact with oral enzymes (Eliades et al., 1999; Schuster et al., 2004). For these reasons Vivera® retainers, which may use a similar material to the one used in Invisalign® aligners, may be characterized by similar properties. When the color stability of Vivera® retainers was tested by immersing them in different staining solutions, it was found that color alteration took place when solutions of coffee, tea and, to lesser extent, red wine were used (Zafeiriadis et al., 2014).

2.3 Plastic toxicity

2.3.1 General aspects

Plastics are used in almost every single aspect of our life such as food containers, household products, water bottles, toothbrushes, computers, telephones, eyeglasses, some clothing, some

toys, etc. The term "plastic" derives from the Greek "plastikos," meaning fit for molding. These popular polymers are now being revealed as potentially possessing adverse health-related effects to human.

One crucial concern regarding the use of plastic-based materials is the leaching of chemical substances called xenoestrogens into the immediate environment surrounding the plastic. Those substances have the ability to produce a biological reaction comparable to that of estrogen hormones. Their action in most cases, takes the form of binding to classic estrogenic receptors (ERs) ERα and ERβ at subtoxic concentrations but capable of inducing estrogenic signals that modify gene expression. This action is called estrogenicity (Quesada et al., 2002; Azarpazhooh, 2008; Zampeli et al., 2012).

One of the materials concerned is Bisphenol-A (BPA) which is produced by the condensation of phenol and acetone in the presence of catalysts and catalyst promoters (Staples et al., 1998). Its molecular formula is $C_{15}H_{16}O_2$ and its structure is shown in Figure 1; the molecular mass is 228.29 g/mol and it exists at room temperature in the form of a white solid with a mild "phenolic" odor (Eramo et al., 2010).

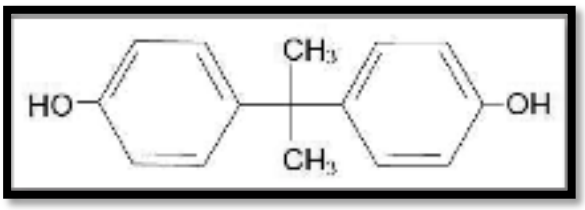

Figure 1. Bisphenol-A chemical structure (Eramo et al., 2010).

BPA is used worldwide in the production of plastic products. It exhibits great similarity in structure with 17β-estradiol and may have similar effects (Azarpazhooh, 2008; Fleisch et al., 2010).

The accumulated level of BPA in the body may vary according to the developmental stage and gender of the subject. Because of the absence of enzymes capable of metabolizing BPA to its biologically inert form, exposure of infants leads to higher BPA body levels relative to those experienced during adulthood. In addition, a sexual dimorphism has been revealed indicating higher plasma BPA levels in male than female fetuses, even after correcting for a positive correlation between body weight and BPA concentration (Schönfelder et al., 2002; Eliades et al., 2007).

According to the United States Environmental Protection Agency (EPA) reference dose and the Food and Drug Administration's acceptable daily intake dose, the presumed ''safe'' dosage is 50 μg/kg/day of BPA (Ritcher et al., 2007; Alonso-Magdelena et al., 2010; Fleisch et al., 2010). However, adverse effects have been documented with BPA doses below the above-mentioned daily level (vom Saal and Hughes, 2005; Welshons et al., 2006; Sekizawa et al., 2008; Bouskine et al., 2009).

The adverse biological effects, demonstrated mainly in experimental animals, are:

a) Hormonal-related effects such as early puberty in females and feminization in males (Timms et al., 2005). In addition, BPA has been shown to be a thyroid hormone receptor antagonist that disrupts the THR-mediated transcription (Zoeller, 2005);
b) Higher risk for breast cancer in females and prostate cancer in males (Timms et al., 2005; Tsai, 2006);
c) Induction of calcium influx, which leads to prolactin release and its associated behavioral effects (Palanza et al., 2002);
d) Development of hyperglycaemia and insulin tolerance (Alonso-Magdalena et al., 2006);
e) Elevation of oxidative stress mediators (Ooe et al., 2005);

f) Upregulation of the cAMP response element-binding factor, which inhibits apoptosis (Quesada et al., 2002);

g) Potential cytotoxic effects such as an immune reaction to material exposure, cell cycle disturbance, cell apoptosis, and induction of mutagenesis or carcinogenesis (Premaraj et al., 2014); and

h) Other biological effects, including neurobehavioral problems such as autism and attention deficit hyperactivity disorder (vom Saal et al., 2007).

When thirteen different intraoral materials were tested in a simulated oral environment, silicone baby bottle nipple samples (20 micrograms) showed BPA leaching after three days of artificial saliva immersion, with no additional leaching thereafter (Sharma et al., 2016). BPA was also found to migrate from polycarbonate water bottles at rates ranging from 0.20 to 0.79 ng/h. This migration of BPA at room temperature was independent of whether or not the bottle had been previously used (Le et al., 2008). Canned food is also a BPA leaching source. According to Takao and co-workers (2002), a low level of BPA was found in water from unheated food cans. This level increased as the heat-treatment temperature increased.

Moreover, more than two million tons of BPA related products are currently produced per year, with an anticipated 6% to 10% annual growth in future demand (Burridge, 2003; Fleisch et al., 2010) which in turn will increase the potential risk from BPA to human health.

2.3.2 Dental environment

In dentistry, the first study to report BPA release in *in-vivo* settings was research by Olea and co-workers (1996) in which salivary BPA levels in patients with dental resin sealants were assessed. This study confirmed the estrogenicity of the sealants used by proliferation tests using human breast cancer cells. As bisphenol A glycidyl methacrylate (Bis-GMA), which is synthesized from

BPA, is incorporated in many resinous materials, it is possible that a BPA residue may remain in Bis-GMA-based resins. Dimethacrylate-based restorative materials may contain BPA as well, as a degradation product from nonspecific esterases and other salivary enzymes that attack the resin matrix and lead to the degradation of BPA derivatives (Soderholm and Mariotti, 1999).

However, due to the diverse conclusions reached by the following studies, a controversy has resulted regarding the actual release of BPA from sealants, and their possible estrogenic action. Some studies have failed to detect any BPA eluted from properly polymerized sealants (Hamid and Hume, 1997; Nathanson et al., 1997; Matasa, 2004). On the other hand, when BPA levels in the urine and saliva of adults treated with two different sealants were compared, there was low level of BPA detected, although these were 1000 times lower than reported elsewhere (Joskow et al., 2006). According to Zampeli et al. (2012) this could result from the incomplete polymerization of sealants caused by the attenuation or scattering of the activating light, the formation of oxygen inhibited layer and/or decreased optical clarity of the sealant itself. The same study found that eluent of a sealant named "Delton Opaque" at a concentration of 10% possessed some estrogenic activity.

2.3.3 Orthodontic materials

The potential estrogenic hazard in orthodontics arises from the fact that the monomers equivalent to those used for dental sealants are also used for the construction of most of orthodontic materials including bonding adhesives, plastic polycarbonate brackets, elastomeric materials and other polycarbonate-made appliances such as aligners (Kloukos and Eliades, 2014).

With regards to the orthodontic adhesive materials used for bonding orthodontic attachments, some investigators believe that the quantity of BPA released from orthodontic adhesives is lower than the threshold required to induce a biologic reaction (Gioka et al., 2005; Eliades et al., 2007).

On the other hand, there are other studies confirming the cytotoxic effects of orthodontic adhesives. An *In-vivo* study which evaluated the cytotoxic effects of six adhesives on the oral mucosa in hamsters proved that the liquid component of one adhesive consistently caused an inflammatory response in all tested animals (Davidson et al., 1982). It has been also shown that activator components of two no-mix adhesives had greater toxicity than other materials (Terhune et al., 1983). Additionally, when the cytotoxic effect of different orthodontic adhesives on human oral fibroblasts was evaluated, it showed that chemically cured liquid paste adhesives were more cytotoxic than light-cured and chemically cured two-paste materials (Tang et al., 1999).

It should be noted that while most research on cytotoxicity in orthodontics has been carried out on monolayer cell cultures (Vande Vannet et al., 2006), when three-dimensional reconstructed human oral epithelium was utilized to determine the toxicity of orthodontic adhesives, architectural and ultrastructural changes in epithelial cells due to penetration of uncured primers were found (Vande Vannet and Hanssens, 2007).

An additional parameter is that in most of the previous studies, the specimens were prepared using the same dimensions as in operative dentistry, something different from the adhesive dimensions in orthodontics (Terhune et al., 1983; Tang et al., 1999). This may affect the amount of monomer release and biocompatibility of these adhesives and provide data not entirely relevant regarding the clinical situation in orthodontics (Gioka et al., 2005). A study to assess the estrogenic action of a chemically cured no-mix and a light-cured orthodontic adhesive resin in a simulated orthodontic environment concluded that there was no evidence of the stimulation of the proliferation of breast cancer cells, indicating the absence of any estrogenicity in the components of orthodontic adhesive eluents (Eliades et al., 2007).

The necessity of ensuring the complete polymerization of the adhesive to decrease the risk of

BPA release has been emphasized by the study of Sunitha et al. (2011). The correlation of BPA release from orthodontic adhesive to different light-curing tip distances was tested. A negative correlation was found between BPA release and light-curing tip distance which means the advisability of keeping the light-curing tip as close to the adhesive as clinically possible in order to ensure the complete polymerization and decrease the chance of BPA release.

As fixed lingual retainers have been extensively used in orthodontic practice to minimize post-treatment changes, the orthodontic adhesives used for bonding such retainers has been tested for possible hazardous BPA release in *in-vitro* and *in-vivo* settings (Eliades et al., 2011 and Kang et al., 2011) respectively. According to the *in-vitro* study, there were measurable amounts of BPA identified in the tested eluents at 10, 20 and 30 days immersion in doubled-distilled water, with the highest levels found in the immersion media of the 1-month groups (2.9 mg/L), whereas the control (tooth storage solution) had 0.16 mg/L (Eliades et al., 2011). The amount of BPA leaching from Bis-GMA–based resin composite used for bonding orthodontic lingual retainers in *in-vivo* condition was found to be low and far below the reference doses for daily uptake (Kang et al., 2011). However, because of some evidence of a "low-dose effect," the amount of BPA released from the resin composites used in orthodontics should not be overlooked (vom Saal and Hughes, 2005; Wozniak et al., 2005).

With regards to other orthodontic materials posing the possibility of BPA release and/or adverse biological effect, polycarbonate brackets have also been investigated. Watanabe and co-workers (2001; 2004) tested the degradation characteristics and the amount of BPA released from new and retrieved polycarbonate orthodontic brackets. It was found that these brackets are capable of producing measurable amounts of BPA. This conclusion was in accordance with the findings of an earlier study (Suzuki et al., 2000).

A recent systematic review regarding the release of BPA from orthodontic materials has concluded that bonding resin had been found to release BPA between 0.85 and 20.88 ng per milliliter *in-vivo*, and from traces to 65.67 ppm *in-vitro*. Polycarbonate brackets released amounts of 22.24 mg per gram in ethanol solution and 697 mg per gram after forty months in water. The included studies were not randomized control trials, and only provided moderate levels of evidence (Kloukos et al., 2013).

Additionally, when different orthodontic materials have been investigated under various thermal and mechanical conditions for the release of BPA, only the thermoformed Biocryl acrylic resin retainer material and a fully cured Transbond XT orthodontic adhesive were found to leach BPA after three days of artificial saliva immersion (Kotyk and Wiltshire, 2014).

2.3.4 Invisalign®

The issue of BPA release has been also addressed with regard to orthodontic aligners (Eliades et al., 2009). It was found that Invisalign® aligners do not have any cytotoxic effect on human gingival fibroblasts and did not show any noticeable estrogenic effects when tested on MCF-7 breast cancer cell line at 5%, 10%, and 20% vol/vol concentration.

Similarly, when Invisalign® aligners were one of the eight orthodontic materials tested by Kotyk and Wiltshire (2014), they could not find any measurable BPA quantity release either.

Recently one study found undesirable effects when epithelial cells were treated with eluates obtained from soaking Invisalign® plastic in saline solution. Some changes were found in viability, membrane permeability, and adhesion of epithelial cells in a saline-solution environment. The secondary results of compromising epithelial integrity might be microleakage and hapten formation which, in consequence, could lead to isocyanate allergy, either systemic or localized to gingiva. This study was the first to report such adverse effect of contact with

Invisalign® plastic on oral keratinocytes (Premaraj et al., 2014).

In contrast to Invisalign® aligners, which are usually used for two weeks almost full-time, Vivera® retainers have been designed for prolonged use, normally on a part time basis. This extended use could lead to degradation and possible deterioration of the material. Since no investigations have dealt with the cytotoxicity and estrogenicity of Vivera® retainers until present, such a study would be a valuable contribution to current knowledge.

SPECIAL PART

3. Research hypothesis

The null hypothesis of this study is that Vivera® retainers, either as-received or after retrieval from patients, have no cytotoxic or estrogenic effect.

4. Aim

The aim of the present study is to investigate the cytotoxicity and estrogenicity of Vivera® retainers by assessing their biological behavioral effect: as-received from the manufacturers, and after retrieval from patients.

5. Materials and Methods

The study sample consisted of six sets of Vivera® retainers, three as-received from the manufacturer and three retrieved from three consecutive patients of the Orthodontic Clinic, Hamdan Bin Mohammed College of Dental Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates, who consented to be included in the study. With regard to the retrieved retainers, these were retrieved after four weeks of use. Each set consisted of a maxillary and a mandibular appliances. This research was approved by the Research and Ethics Committee of Hamdan Bin Mohammed College of Dental Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences (Ref: HBRCDM/EC/2003).

The evaluation of cytotoxicity and estrogenicity of all retainers took place in the Laboratory of Cell Proliferation and Ageing, Institute of Biosciences and Applications, National Center for Scientific Research "Demokritos", Athens, Greece. The retainers were transferred from Dubai by one member of the research team in such a way that their physical condition remained unchanged during transfer, after retrieval or delivery, respectively. All retrieved retainers were divided in two equal parts randomly regardless of being upper or lower component. Each one subjected to either mode of sterilization procedures, i.e. gamma-irradiation (IRR) or autoclaving (AUTOCL). The as-received retainers were divided into three equal parts randomly as well. Two parts were sterilized, with each part using one of the above-mentioned procedures, while the third part of as-received retainers was not subjected to any sterilization mode, so as to test the effects of the sterilization procedure.

Following sterilization, all samples were immersed in sterile normal saline (NaCl 0.9% w/v) with each sample in different container and incubated for fourteen days at 37 °C. Normal saline (NS) without any retainer was incubated under the same conditions in parallel, to be used as negative

control. All retainers, which were following specific allocation and procedures of sterilization (Table 1), were aliquoted and kept at -20 °C to maintain its integrity until further experimental use. Samples obtained from incubation of as-received/unsterilized retainers (i.e. samples 2 and 4) were considered to be identical (Table 1).

Table 1. Sample allocation and procedures of sterilization.

Sample No.	Patient code	Used retainer	Sterilization procedure
1	ALMFA014	No	IRP
2	ALMFA014	No	No
3	ALMAS000	No	AUTOCL
4	DOCTR000	No	No
5	DOCTR000	No	AUTOCL
6	DOCTR000	Yes	IRP
7	DOCTR000	Yes	AUTOCL
8	ALMAS000	No	IRP
9	ALMAS000	Yes	IRP
10	ALMAS000	Yes	AUTOCL
11	ALMFA014	Yes	IRP
12	ALMFA014	Yes	AUTOCL

The estrogenicity assays involved 2 cell lines, i.e. the estrogen-sensitive MCF-7 and the estrogen-insensitive MDA-MB-231 (both from human breast adenocarcinoma), in order to exclude the possibility that a decreased proliferation of cells induced by the retainer eluent would mask a potential induction of proliferation due to estrogenicity.

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), at 37 °C, in 5% carbon dioxide, in a humidified incubator and regularly subcultured by using trypsin-citrate solution. To evaluate the estrogenicity of the samples, the cells were plated in 48-well flat-bottomed microwells (10,000 cells per well) in DMEM and 10% fetal calf serum. Twenty-four hours later, the medium was changed to phenol-free DMEM supplemented with 2% fetal calf serum pretreated with dextran-coated charcoal, along with the solutions to be tested, at concentrations 5%, 10% and 20% vol/vol. β- Estradiol (βE_2) was used as positive control, and NS solution was used as negative control. After six days of incubation, with medium renewal at day three, the cells were detached using trypsin-citrate solution and counted in a Z1 Beckman- Coulter counter. Assays were performed in triplicate, and the results were averaged.

6. Statistics

The statistical analysis of data was performed with 2-way analysis of variance (ANOVA) with appliance and concentration as predictors. Differences were further investigated with the Tukey multiple comparison test at the 0.05 level of significance.

7. Results

An initial experiment was performed using samples 1-3, corresponding to as-received retainers, to assess the effects of the two sterilization procedures while the third one served as a control. As shown in Figure 2, none of the samples, at any concentration tested, induced the proliferation of MCF-7 cells compared to the negative control, in contrast to the pronounced stimulation by all three β-estradiol concentrations (within the physiological limits) tested. (See also Appendix table 1).

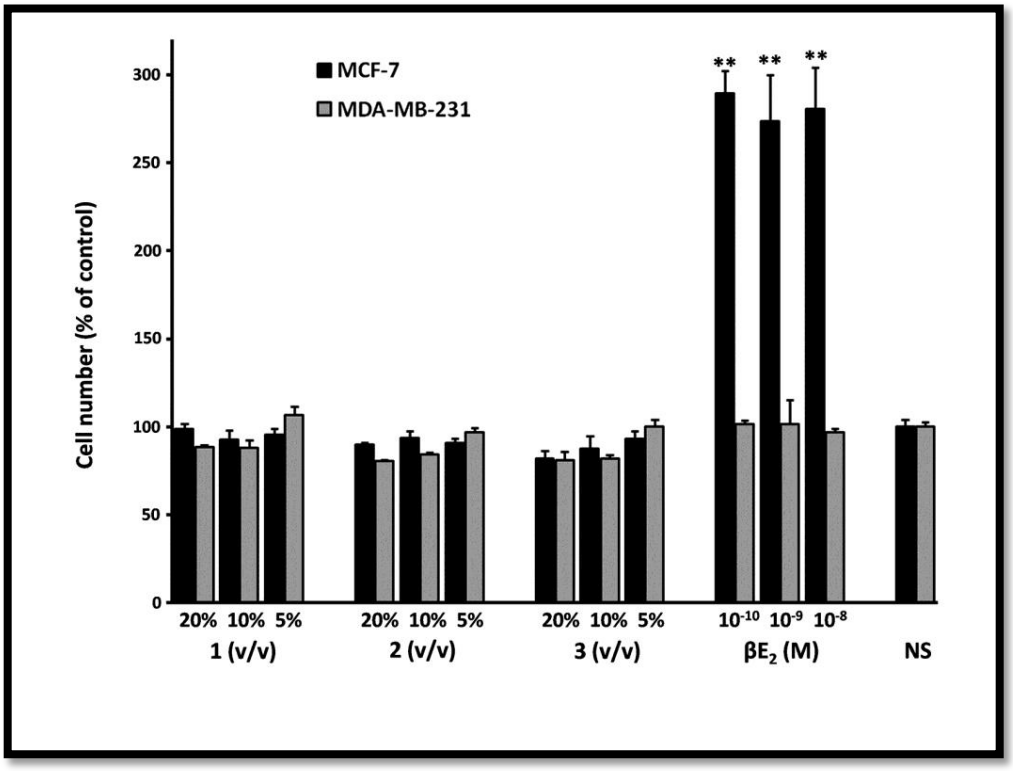

Figure 2. Proliferation of MCF-7 vs. MDA-MB-231 cells in response to retainer eluent samples: effect of sterilization procedure.

However, as shown in Figure 3, after gamma-irradiation, the appearance of the retainers was altered, acquiring a yellowish color reminiscent of the effect of ultraviolet light on plastic materials. Hence, it was considered that the sterilization through gamma-irradiation could potentially damage the plastic, and autoclaving was finally chosen as the preferred mode of sterilization.

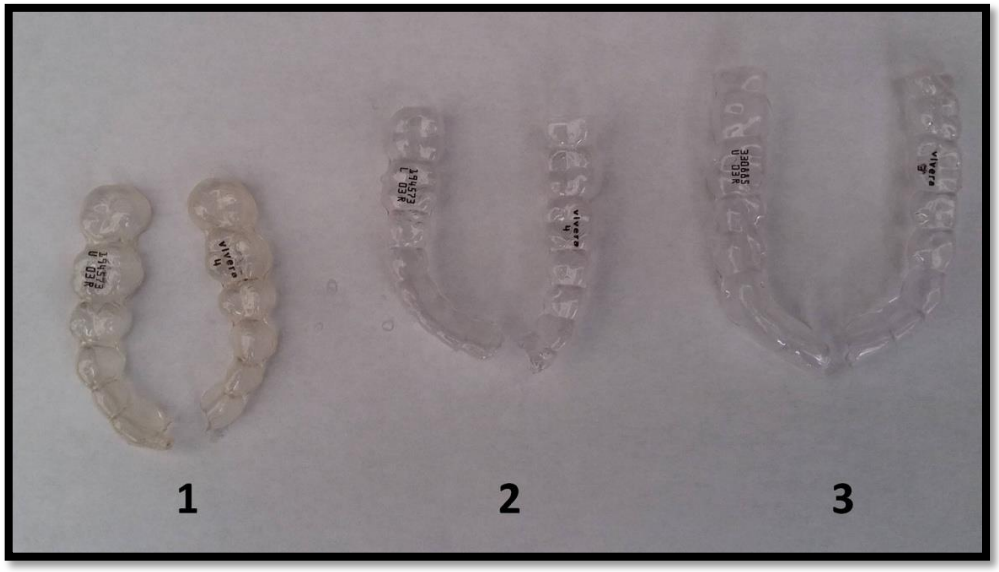

Figure 3. Appearance of the retainers after sterilization: (1) sterilized through gamma-irradiation; (2) non-sterilized; and (3) sterilized through autoclaving.

Accordingly, samples 7, 10, and 12, corresponding to retrieved retainers form the three patients were evaluated in comparison to samples from as-received retainers (either autoclaved or not, i.e. samples 4 or 5).

As shown in Figure 4, no significant MCF-7 proliferation was induced by the samples 7, 10, and 12, compared either to the eluents from as-received retainers, i.e. 4 and 5, or to the negative control. As expected, β-estradiol induced a potent stimulation of MCF-7 cell proliferation, while

no effect was observed on MDA-MB- 231 cells. (See also Appendix table 2).

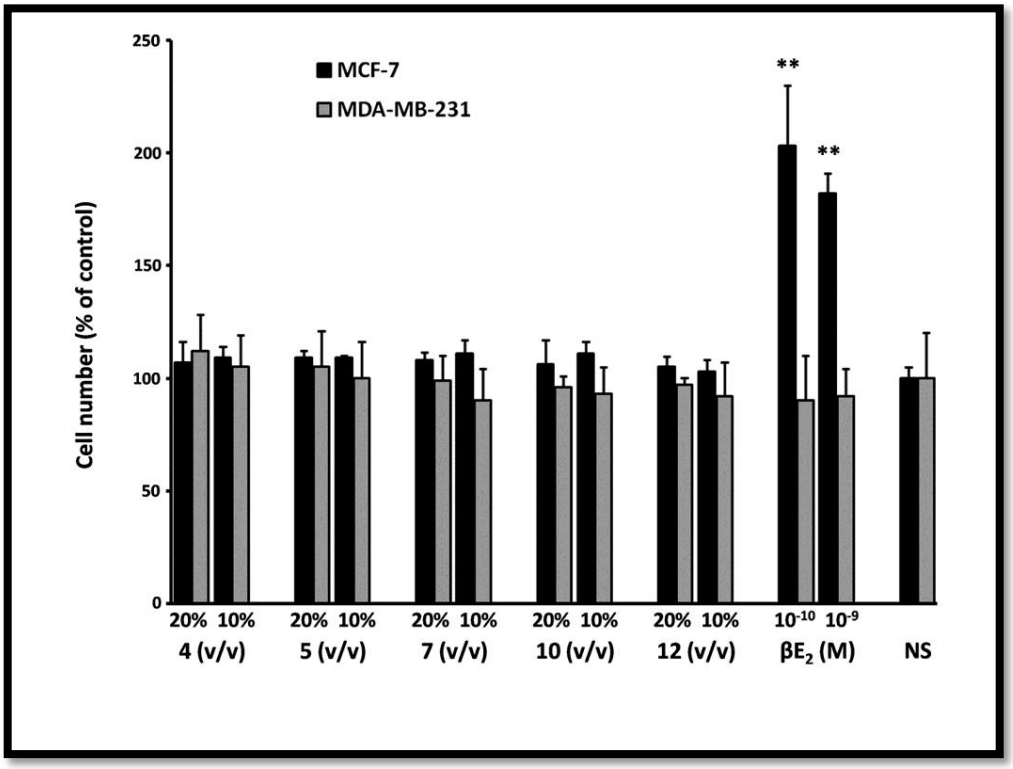

Figure 4. Proliferation of MCF-7 vs. MDA-MB-231 cells in response to retainer eluent samples (average from two experiments).

Thus, the null hypothesis was accepted meaning that Vivera® retainers either the as-received or after retrieved from patients have no cytotoxic or estrogenic action.

8. Discussion

This study was designed to test the cytotoxic and estrogenic behavior of Vivera® retainers either as-received or after use by the patients for a four week time period. The results failed to reveal any measurable adverse biological activity from either category, as-received or retrieved. The first possible explanation could lie in the stability of material used for the fabrication of Vivera® retainers. It is not stated as different from Invisalign® aligner material, which is a polyurethane-derived one. This material is manufactured from polyether urethanes used as raw material and as described by Eliades et al. (2005) has the following structure; "*These polymers* have *short rigid portions (the aromatic rings and the ureas) joined by short flexible ''hinges'' (the diamine linker and the CH_2 group between the aromatic ring) and long very flexible portions (the polyether)".* One should note that the chemical composition of this material as described does not contain the necessary ingredients to release BPA and induce its known adverse biological effects. However, it had been shown that some materials exhibit estrogenicity despite not containing BPA in their composition (Zampeli et al., 2012).

Another reason could be the relatively short duration of the experiment as the used retainers were retrieved after only four weeks. It should be noted that the duration of retention varies and depends on many factors including: type of initial malocclusion, treatment mechanics, patient compliance, facial growth pattern, and cosmetics aspects. With regards to Vivera® retainers, the company always supplies patients with three sets (including both upper and lower appliances) since it is anticipated that during the retention period a need of replacement may occur. Yet, there is no definitively specified retention period recommended for patients using Vivera® retainers. Furthermore, the incubation period was for two weeks only. In the present experimental set-up, the fact that during those two weeks, the normal saline immersion solution was left without being renewed should be taken into consideration. Accordingly, any effect

likely to occur would be expected to be concentrated and amplified compared to the oral environment where saliva plays a role in diluting and renewing the medium as well as providing some protection effect (Premaraj et al., 2014).

The estrogenicity of the eluent from the materials tested was measured using an established assay in protocol for estimating the proliferation of the estrogen-responsive MCF-7 cell line. These cells are known to express estrogen receptor-α, which is important for the proliferative effect of estrogens. It was proposed on account of its known intense proliferation upon exposure to very low levels of estrogens and therefore chosen for this sensitivity (Soto et al., 1986). In addition, it is derived from human breast cancer tissue, so the results will be more directly relevant to humans. Primarily, the cells were used to assess estrogenic action through the effect of increasing the mitotic indexes of rodent epithelia (Al-Hiyasat et al., 2004). However, rat hepatic microsomes have been found to be more effective in reducing estrogenicity compared to the human liver (Elsby et al., 2001).

On the other hand, using an estrogen insensitive cell line, MDA-MB-231 to serve as a sham control was essential. This sham control aided to a more precise estimation of the estrogenicity of the tested materials as it excluded the possibility that the estrogenic proliferative effect could be masked by the inhibitory cytostatic action of the eluents.

17ß-estradiol is a natural hormone used in this study as a positive control. This hormone is known to induce effects at concentrations much lower than the levels at which all hormone receptors become bound. The significance of this is that once all receptors have been occupied, further increases in the level of the hormone do not result in a correspondingly increased response. Therefore, the lack of response to excessively high concentrations of effectors could be misinterpreted as lack of effect (Welshons et al., 2003). That was also excluded in this study, as

the maximum concentration of the eluents from retainers was 20% vol/vol, and this was considered adequate.

Including used retainers in the study was considered a strength, because it took into consideration the possibility that some material might react differently in the oral environment in terms of degradation and changes in physical and biological properties. The present study addressed this possibility by testing both conditions of the retainers: as-received and retrieved from patients. Sample size was not considered an important issue in the current study because the aim was to test the cytotoxic and estrogenic reactions of the material itself without correlating it to human factors. However, human parameters such as developmental stage, gender, habits, etc. may play a role in varying the estrogenic effect of a material and can be considered in further studies.

All the retrieved retainers were subjected to one of the sterilization procedures, gamma irradiation or autoclaving, in order to eliminate any possibility of bacterial growth masking the results of the estrogenicity assay. Gamma-irradiation sterilized retainers, samples 6, 8, 9, and 11, were then excluded from the experiment as its appearance was distorted, getting a yellowish color, suggestive of the effect of ultraviolet light on plastic materials.

This was the first study conducted to measure these parameters; the cytotoxic and estrogenic effect of this type of retainers. To the best of our knowledge, no other studies have been published dealing in a comparable way with the testing of the biological behavior of such material utilizing similar methodological processes apart from one study concerned only with as-received Invisalign® aligners, which arrived at similar results regarding the as received retainers (Eliades et al., 2009).

The limitation of the current study lies in the results being based on *in-vitro* assessment.

However, *In-vivo* testing is not always feasible because of the ethical and practical difficulties involved, such as the time required, difficulties in controlling the confounding variables, and frequent problems in interpreting the results. However, the use of the cell cultures with a human origin in this study constituted an advantage (Pratsinis et al., 2013).

9. Conclusions

Based on the results of this study, carried out to test the cytotoxic and estrogenic behavior of both as-received and retrieved Vivera® retainers, it can be concluded that there is no significant release of substances with estrogenic activity after the incubation of these retainers, as-received or retrieved, in normal saline for two weeks at body temperature.

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11. Appendices

Appendix table 1. Proliferation of MCF-7 vs. MDA-MB-231 cells in response to retainer eluent samples: effect of sterilization procedure.

Sample No.	MCF-7				MDA-MB-231			
	Concentration	Mean (cells/well)	SD	*t*-test (P-value)	Concentration	Mean (cells/well)	SD	*t*-test (P-value)
1.	20%	82760	2742	0.68710915	20%	85295	940	0.00129523
	10%	80255	4748	0.20725934	10%	84705	4380	0.00693170
	5%	81435	2995	0.45953386	5%	98000	4503	0.13161910
2.	20%	75440	1082	0.01540441	20%	77400	1016	0.00006886
	10%	81220	3286	0.24240784	10%	81005	1441	0.00026766
	5%	77470	2280	0.15060040	5%	88755	2648	0.35440391
3.	20%	68690	3936	0.00294624	20%	77785	5108	0.00111953
	10%	75760	6294	0.07056464	10%	78915	2218	0.00020642
	5%	79315	3767	0.27641554	5%	92140	3340	0.92147468
NS.	20%	83960	4969		20%	96270	3756	
	10%	86580	7587		10%	96310	3754	
	5%	85240	9154		5%	91810	5483	
βE_2	βE_2 10^{-10}	247610	11030	0.00000014	βE_2 10^{-10}	90145	2024	0.46327282
	βE_2 10^{-9}	234060	22523	0.00001258	βE_2 10^{-9}	90115	12539	0.85763748
	βE_2 10^{-8}	240030	20296	0.00000552	βE_2 10^{-8}	86275	1812	0.12780279

Appendix table 2. Proliferation of MCF-7 vs. MDA-MB-231 cells in response to retainer eluent samples (1st and 2nd experiment).

Sample No.	MCF-7				MDA-MB-231			
	Concentration	Mean (cells/well)	SD	t-test (P-value)	Concentration	Mean (cells/well)	SD	t-test (P-value)
4.	1st 20%	78500	5655	0.126240	1st 20%	73393	2059	0.000581
	2nd 20%	79067	4167	0.835549	2nd 20%	78767	1890	0.617280
	1st 10%	97980	2359	0.001988	1st 10%	76533	6432	0.065221
	2nd 10%	98000	2605	0.159620	2nd 10%	89107	4447	0.154361
5.	1st 20%	77133	2022	0.099476	1st 20%	68707	3633	0.013257
	2nd 20%	83680	317	0.034135	2nd 20%	73053	2707	0.034964
	1st 10%	93867	4460	0.065718	1st 10%	73867	2217	0.017934
	2nd 10%	102727	6647	0.104356	2nd 10%	82900	5014	0.022984
7.	1st 20%	76533	3110	0.138409	1st 20%	63267	8499	0.457933
	2nd 20%	83433	4383	0.172583	2nd 20%	71120	3621	0.030276
	1st 10%	99853	2470	0.001253	1st 10%	66013	1464	0.768971
	2nd 10%	99700	5074	0.164052	2nd 10%	75207	3861	0.001624
10.	1st 20%	78680	985	0.056028	1st 20%	59053	1701	0.921252
	2nd 20%	76667	1716	0.414519	2nd 20%	72653	2450	0.020634
	1st 10%	99513	3426	0.004050	1st 10%	67260	4007	0.799564
	2nd 10%	100513	8078	0.247323	2nd 10%	79560	4518	0.006931

Appendix table 2. Continued.

Sample No.	MCF-7				MDA-MB-231			
	Concentration	Mean (cells/well)	SD	t-test (P-value)	Concentration	Mean (cells/well)	SD	t-test (P-value)
12.	1st 20%	75167	3553	0.220540	1st 20%	58687	2032	0.742616
	2nd 20%	79867	2232	0.528121	2nd 20%	74087	2308	0.044386
	1st 10%	92793	2152	0.015928	1st 10%	68360	2252	0.391149
	2nd 10%	93247	3039	0.896529	2nd 10%	76027	6109	0.008224
NS.	1st 20%	69333	5992		1st 20%	59187	1387	
	2nd 20%	78420	2864		2nd 20%	78133	735	
	1st 10%	87200	1091		1st 10%	66527	2420	
	2nd 10%	93620	3544		2nd 10%	93993	1876	
βE_2	1st 20%	βE_2 10^{-10}	138167	4497	0.000091	1st 20%	βE_2 10^{-10}	62133	2685	0.166502
		βE_2 10^{-9}	124047	22876	0.016032		βE_2 10^{-9}	59180	1826	0.996223
	2nd 20%	βE_2 10^{-10}	135780	7156	0.000209	2nd 20%	βE_2 10^{-10}	69520	728	0.000134
		βE_2 10^{-9}	148553	15750	0.001618		βE_2 10^{-9}	68927	1946	0.001558
	1st 10%	βE_2 10^{-10}	193827	27254	0.002483	1st 10%	βE_2 10^{-10}	69080	4937	0.466255
		βE_2 10^{-9}	163633	1056	0.000000		βE_2 10^{-9}	66640	485	0.940433
	2nd 10%	βE_2 10^{-10}	172340	6746	0.000057	2nd 10%	βE_2 10^{-10}	71493	8306	0.010208
		βE_2 10^{-9}	164367	14492	0.001198		βE_2 10^{-9}	78547	3570	0.002679

INFORMED CONSENT

PATIENTS' INFORMED CONSENT AND AGREEMENT REGARDING THEIR PARTICIPATION IN THE RESEARCH PROJECT USING VIVERA® RETAINERS:

Your orthodontist has recommended Vivera® retainers for your retention course after orthodontic treatment.

DEVICE DESCRIPTION:

The Vivera® retainer developed by Align Technology, Inc. This is a clear plastic removable appliance that will keep your teeth in their achieved position. It is a customized product that is produced specifically for your personal benefit using sophisticated computer graphics technology.

BENEFITS:

- Vivera® retainer offers an esthetic alternative to conventional types of retainers.
- Vivera® retainer is nearly invisible so many people won't realize you are wearing it.
- Vivera® retainer allows for normal brushing and flossing tasks that are impaired by some other conventional types of retainers.
- Vivera® retainer does not have metal wires associated with other conventional types of retainers.
- Vivera® retainer may improve oral hygiene habits.

RISKS AND INCONVENIENCES:

The use of Vivera® retainer may involve some of the risks outlined below:

- Teeth have tendency to return to their original position after treatment. Failure to wear the retainer as recommended by your orthodontist will adversely affect our achieved results.
- Vivera® retainer may temporarily affect speech and may result in a lisp, although any speech impediment caused by the retainer should disappear within one or two weeks.
- Vivera® retainer may cause a temporary increase in salivation or mouth dryness and certain kinds of medication can heighten this effect.
- Gums, cheeks and lips may be scratched or irritated.
- Tooth decay, periodontal diseases, inflammation of the gums or permanent markings (e.g. decalcification) may occur if patients consume food or beverages containing sugar, do not brush and floss their teeth properly before wearing the retainer, or do not use proper oral hygiene and preventive maintenance.
- In rare instances, slight superficial surface wear of the retainer may occur where patients may habitually grind their teeth or where the teeth may rub against the plastic. Generally, this is not a problem as overall retainer integrity and strength remain intact.
- Vivera® retainer breakage is more likely in patients with a severe grinding habit.
- Vivera® retainer or parts thereof may be accidentally swallowed or aspirated.

- Allergic reaction may occur.
- In rare instances, patients with hereditary angioedema (HAE), a genetic disorder, may experience rapid local swelling of subcutaneous tissue including the larynx, HAE may be triggered by mild stimuli including simple dental procedures.

I have been given adequate time to read this content form. I have been sufficiently informed and understand the benefits, risks of Vivera® retainer. I have had the opportunity to ask questions and discuss any concerns about the use of such type of retainer.

I authorize my orthodontist to use my medical records including, but not limited to, radiographs (x-rays), reports, charts, medical history, findings, plaster model, photographs, impressions or intra oral scans of teeth, diagnosis, medical testing, test results, billing, and other treatment records with other licensed orthodontists, and for educational and research purposes.

I understand that the use of my medical records may result in disclosure of my "individually identifiable health information" as defined by Health Insurance Portability and Accountability Act (HIPAA). I hereby consent to the disclosure(s) as set forth above. I will not, nor shall anyone on my behalf seek legal, equitable or monetary damage or remedies for such disclosure. I acknowledge that use of my medical records is without compensation and that I will not nor shall anyone on my behalf have any right of approval, claim of compensation, or seek or obtain legal, equitable or monetary damages or remedies arising out of any use such that comply with the terms of this consent.

A photostat copy of this consent shall be considered as effective and valid as an original.
I have read and understand and agree to the terms set forth in this consent as indicated by my signature below.

Patient name Signature

--- ---

Witness name Signature

--- ---

Dr. Dimitris Kletsas
Research Director
Laboratory of Cell Proliferation & Ageing
Institute of Biosciences & Applications
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Athens, 15 December 2016

To: Whom it may concern

This is to certify that Dr. Shaima Rashid Al Naqbi, orthodontic resident, Hamdan Bin Mohammed College of Dental Medicine, MBRU, visited the Laboratory of Cell Proliferation and Ageing, in the Institute of Biosciences and Applications of the National Centre for Scientific Research "Demokritos" from 13/4/2016 to 15/4/2016. During her visit she was trained and worked on different methods of cell biology, such as cell culture methodology, as well as viability, proliferation and oestrogenicity assays, within the framework of her PhD thesis.
A publication based on this work is currently under preparation, as to be submitted in a peer-reviewed journal.

Sincerely,

The Head of the Laboratory
Dimitris Kletsas, Research Director