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ORIGINAL RESEARCH

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Bisphenol A release from commercially available 3-dimensionally printed resins and human cell apoptosis to bisphenol A: an *in-vitro* study

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Abstract

Bisphenol A (BPA) from dental materials may be linked to children's health issues. This study aimed to assess the release of BPA from commercially available 3-dimensional (3D)-printed resin materials and evaluate BPA-related apoptotic effects on human periodontal ligament cells and gingival fibroblasts. Commercially available 3D-printed resin materials for prosthodontic use were selected as follows: NextDent C&B MFH (3D Systems, Rock Hill, SC, USA), DIOnavi-P. MAX (Dio Co., Busan, Korea), and DIOnavi-Denture02 (Dio Co., Busan, Korea). Identical cuboidal samples (1 cm $\times$ 1 cm $\times$ 0.5 cm) were printed from the materials and cured. BPA release was assessed using liquid chromatography/mass spectrometry (LC/MS). In addition, human gingival fibroblasts and periodontal ligament cells were exposed to various BPA solutions based on the LC/MS results. Cell Counting kit-8 (CCK-8) and real-time polymerase chain reaction analyses were performed to evaluate BPA-related apoptotic effects. The LC/MS analysis confirmed that none of the 3D-printed resin materials released BPA after curing. Both human gingival fibroblasts and periodontal ligament cells showed lower viability after BPA exposure. Regarding apoptosis-related gene expression, *Caspase10* (*CASP10*) expression in periodontal ligament cells was significantly different in the BPA solutions ($p < 0.05$). The expression of BAX and *Capspase8* (*CASP8*) in gingival fibroblasts was significantly increased by BPA in a dose-dependent manner ($p < 0.05$). Within the limitations of this study, the 3D-printed resin materials were not found to release BPA. This finding implies that 3D-printed resin materials are not associated with potential BPA-related risks in children.

Keywords

3-dimensional printing; Bisphenol A; Children; Dental materials; Digital dentistry; Pediatric dentistry

1. Introduction

The advent of 3-dimensional (3D) printing technology has been expected to drive industrial innovation through technological paradigm shifts in various fields. 3D printing technology enables new industrial forms through the convergence of manufacturing, information and communication technology, and personalized production. The adoption of 3D printing technology in the field of dentistry has been associated with a number of challenges. Due to the rapid production, high precision, personal customization, and easy application of 3D printing technology, it has gained widespread acceptance in dentistry [1]. Moreover, 3D printable polymers are increasingly used for dental restorations in the field of restorative dentistry [2, 3]. However, some environmental concerns remain regarding pollution from 3D printer-related microparticles because 3D printers form a specific shape by dissolving the printing materials [4].

Bisphenol A (BPA) is a chemical used in various consumer products. BPA may be linked to problems with genital development, immune function, thyroid function, and neurodevelopment in children [5]. BPA exposure in children may cause cardiovascular disorders by altering the structure and function of the heart [6]. A previous human study confirmed a link between urinary BPA levels and self-reported cardiovascular disease in children [7]. As BPA is an endocrine disruptor, early BPA exposure in childhood may increase adiposity and the risk of altered pubertal development by affecting reproductive hormones [8]. Furthermore, previous studies demonstrated that gestational BPA levels of children are associated with

aggressive and hyperactive behaviors [8, 9]. The primary source of BPA is environmental pollution [10]. However, BPA exposure from dental materials still remains a concern [5]. BPA derivatives, such as Bisphenol A-glycidyl methacrylate (Bis-GMA), are one of the main components of composite resins as a monomer. The possibility of human exposure may exist after composite resin restorations because the monomer cannot be perfectly polymerized during light curing. Previous studies confirmed the release of BPA after composite resin restoration [11, 12]. A previous study evaluated BPA release from dental sealants and orthodontic bonding agents, such as Band-Lok® and Transbond^{TM} PLUS and showed that BPA was still released from the materials after they were cured [13].

However, few studies have examined the release of BPA from 3D-printed resin materials. 3D-printed resin materials are progressively being employed for full-coverage restorations in the field of pediatric dentistry [2, 3]. Because the volume and surface area of 3D-printed resin materials are greater than those of composite resin restorations, it is essential to assess the release of BPA from 3D-printed resins in terms of biocompatibility. Therefore, this study aimed to investigate the release of BPA from commercially available 3D-printed resin materials. The null hypothesis of this study was that there is no difference between BPA levels released from commercially available 3D-printed resin materials and composite resins.

2. Materials and methods

2.1 Materials

The materials tested are listed in Table 1.

TABLE 1. Materials used in this study.

Product	Manufacturer	Uses
NextDent C&B MFH (Micro Filled Hybrid)	3D systems, Rock Hill, SC, USA	Crown & bridges
DIOnavi-P. MAX	Dio co., Busan, Korea	Crown & bridges
DIOnavi-Denture02	Dio co., Busan, Korea	Dentures
Filtek Z250	3M ESPE, St.Paul, USA	Direct restorations

2.2 Liquid chromatography/mass spectrometry (LC/MS)

BPA release was assessed using liquid chromatography/mass spectrometry (LC/MS) [14]. First, BPA solutions (0.1, 1, 10, and 100 ppm) were prepared by diluting pure BPA powders (Product number: 239658, Sigma-Aldrich, Milwaukee, WI, USA) in distilled water (DW) for BPA identification and quantification (Fig. 1). BPA identification in the solutions was performed using an Ultimate 3000 RSLC nano HPLC system with a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Mass Spectrometer, Thermo Scientific, USA). According to International Organization for Standardization (ISO) standard 10993-12, all chromatographic separations were performed with a column (Acquity UPLC BEH C18; Waters Corp., Milford, MA, USA) with a dimension of 2.1 mm × 100 mm, 1.7 μm. The mobile phase consisted of water with 0.01% formic acid (mobile phase A) and acetonitrile/methanol with 0.01% formic acid (mobile phase B). The column temperature was maintained at 45 °C. Furthermore, 6.5 mM AB reagent in DW and 6.5 mM AB in acetonitrile were used as solvents. Five microliters of each solution was injected at 5 °C. Also, a 4-point standard curve with BPA concentrations was constructed to estimate the samples of the test materials based on these solutions.

Identical cuboidal samples (1 cm × 1 cm × 0.5 cm) of the 3D-printed resin materials were printed and cured using a 3D printer (DIO PROBO Z; Dio Co., Busan, Korea). Samples of Filtek Z250 were manually made with the same dimensions. Filtek Z250 was placed twice in a silicone mold (1 cm × 1 cm × 0.5 cm) with a thickness of 2.5 mm. A SmartLite Focus (Dentsply Sirona, Milford, DE, USA) was used to perform incremental curing in each placement according to the manufacturer's protocol. Seven samples per group were then placed in a conical tube containing 8 mL of DW. The samples were incubated for one and seven days at 37 °C. Three conical tubes were allocated to each material (n = 3 per group). The identification of BPA from the samples was performed using LC/MS in the same way as for the BPA solutions.

2.3 BPA-related cell toxicity by CCK-8 assay

BPA solutions (0, 0.001, 0.01, and 0.1 ppm) were prepared by diluting with methyl alcohol. Human gingival fibroblasts (HGF) and periodontal ligament (PDL) cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Grand Island, NY) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO_2. Cells at passage 4 were used. The cell toxicity was evaluated using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) as previously described [15]. The cells were seeded in a 96-well plate with 100 μL of culture medium. After one day of incubation, the medium was removed, and 90 μL BPA solution was added. The cells were then exposed to BPA solutions for 0.5, 1, 6, and 24 h. Then, 10 μL of CCK-8 solution was added to the plate and incubated for 3 h at 37 °C. Cell viability was determined by measuring optical density at 450 nm using a Benchmark Plus Multiplate Spectrophotometer (Bio-Rad, Hercules, CA, USA).

2.4 Apoptosis-related gene expression by real-time polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed to investigate apoptosis-related gene expression as previously described with some modifications [16, 17]. HGF and PDL cells were seeded in 6-well plates with 2 mL of culture medium. After one day of incubation at 37 °C and 5% CO_2, BPA solutions (0, 0.001, 0.01 ppm) were added to plates for 6 h. Cell pellets were acquired after the dissociation of the cell suspensions using a 0.25% trypsin-EDTA solution (Gibco). RNA was isolated and converted to complementary DNA using reverse transcriptase

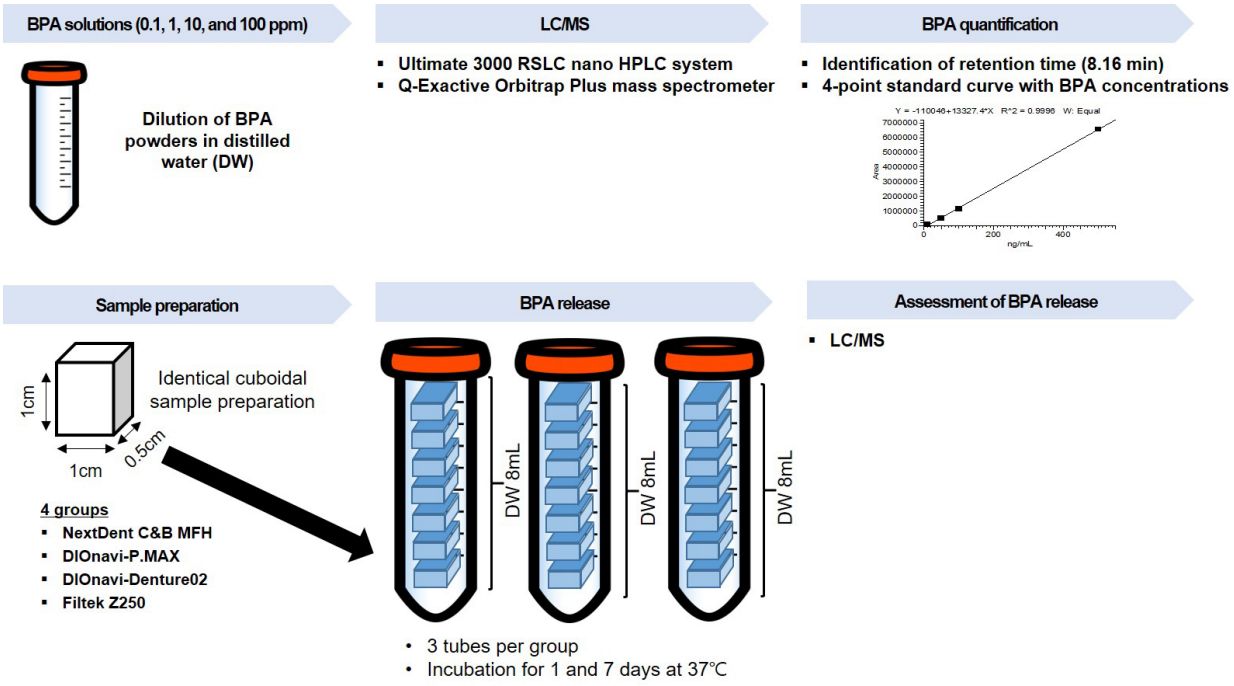

FIGURE 1. Illustration of the methodology. Bisphenol A (BPA) was diluted with distilled water (DW). BPA solutions with 0.1, 1, 10, and 100 ppm were evaluated using liquid chromatography/mass spectrometry (LC/MS). The retention time of BPA was determined to be 8.16 min. BPA quantification was performed by constructing a 4-point standard curve with BPA concentrations. Four groups of identical cuboidals were made (NextDent C&B MFH, DIOnavi-P.MAX, DIOnavi-Denture02, and Filtek Z250). To simulate BPA release, 7 cubiodals were placed in a conical tube with 8 mL of DW. Three conical tubes were allocated to each group. Finally, LC/MS was used to assess BPA release.

(SuperScript II Reverse Transcriptase; Invitrogen, Carlsbad, CA, USA). PCR conditions were set up at 60 °C annealing with 2X Power SYBR Green Master Mix (AB) reagent. RT-PCR was performed using the StepOnePlus RT-PCR system and measured by SYBR green fluorescence using Power SYBR Green Master Mix (Thermo Fisher Scientific, Carlsbad, CA, USA) under the following conditions: 15 min denaturation at 95 °C, followed by 40 amplification cycles of denaturation for 15 s at 95 °C, and annealing for 30 s at 59 °C. Complementary DNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels using the $2^{-\Delta\Delta Ct}$ method. The primers were selected as described in previous studies [18, 19]; *B-cell lymphoma 2* (*BCL2*), *BCL2 associated X* (*BAX*), *Capspase8* (*CASP8*), and *GADPH* (as a housekeeping gene).

2.5 Statistical analysis

Gene expression data with BPA solutions were statistically analyzed using the Kruskal-Wallis test, followed by the Mann-Whitney test as a post hoc test ($\alpha = 0.017$), in SPSS software (version 21.0; IBM Corp., Armonk, NY, USA). Statistical significance was indicated at $p < 0.05$.

3. Results

3.1 Identification of BPA from 3D-printed resin materials

The release of BPA was only identified from Filtek Z250 samples on both one and seven days (12.16 ± 1.96 ng/mL and 12.92 ± 1.68 ng/mL on days one and seven, respectively). BPA release was not observed in NextDent C&B MFH, DIOnavi-P. MAX, and DIOnavi-Denture02, respectively (Table 2). Fig. 2 shows representative images of the LC/MS analysis of each group on day seven.

TABLE 2. Identification of Bisphenol A from the 3D-printed resin materials.

Product	Day	BPA release (ng/mL)
NextDent C&B MFH (Micro Filled Hybrid)	1	ND
NextDent C&B MFH (Micro Filled Hybrid)	7	ND
DIOnavi-P. MAX	1	ND
DIOnavi-P. MAX	7	ND
DIOnavi-Denture02	1	ND
DIOnavi-Denture02	7	ND
Filtek Z250	1	12.16 ± 1.96
Filtek Z250	7	12.92 ± 1.68

ND: Not detectable; BPA: Bisphenol A.

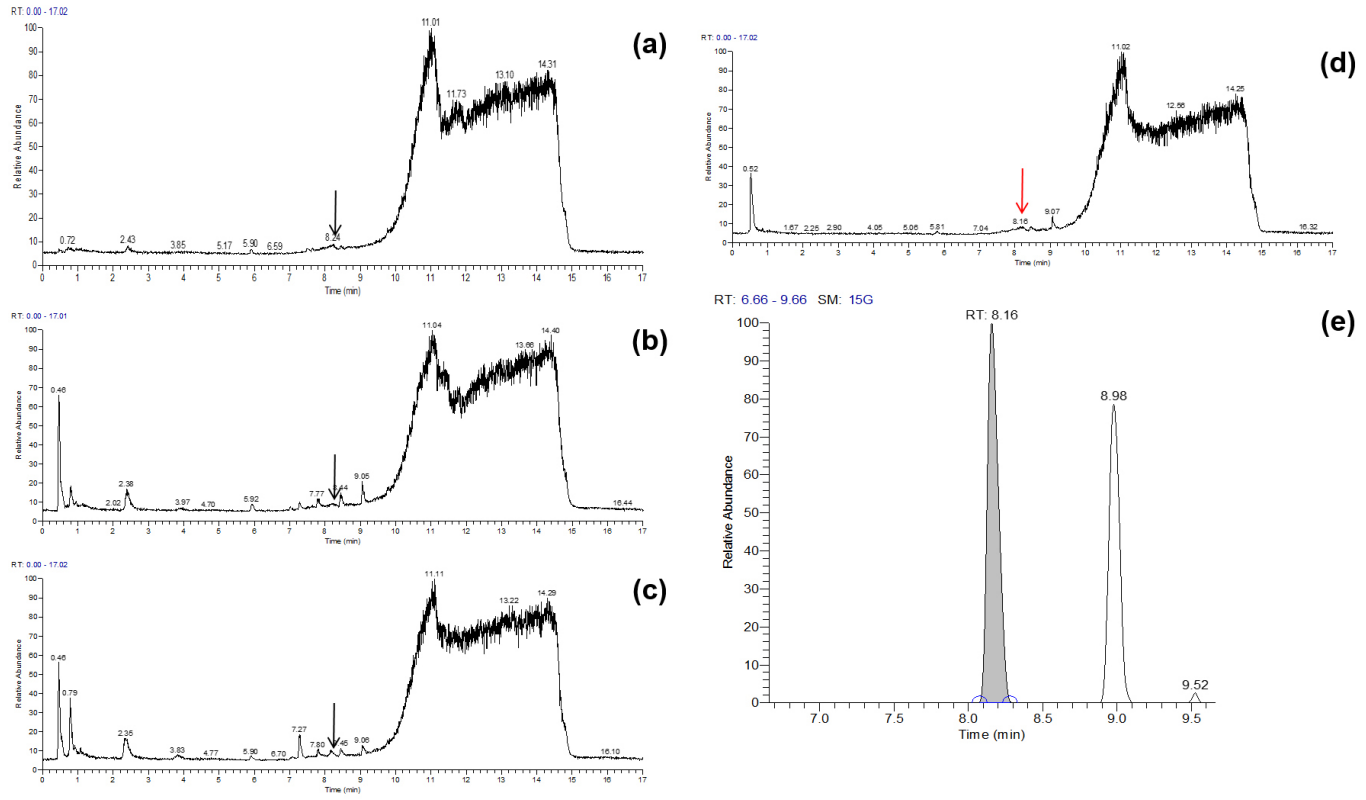

FIGURE 2. LC/MC chromatogram images of 3D-printed resin materials. (a) NextDent C&B MFH (Micro Filled Hybrid) at day seven, (b) DIOnavi-P. MAX at day seven, (c) DIOnavi-Denture02 at day seven. Note that no peak is visible (black arrows). (d) Filtek Z250 at day seven. Note that the peak is visible (a red arrow). (e) Identification of BPA. BPA release from Filtek Z250 at day seven based on the predetermined retention time (8.16 min) of BPA.

3.2 BPA-related cell toxicity and apoptosis-related gene expression

Fig. 3 shows the BPA-related toxicity in HGF and PDL cells. The cell viability generally decreased in a time-dependent manner.

Regarding apoptosis-related genes, only CASP10 expression in PDL cells was different among the BPA solutions ($p < 0.17$). There were no significant differences in *BCL2, BAX* or *CASP8* gene expression in PDL cells (Fig. 4).

Fig. 5 shows the expression of apoptosis-related genes in HGF. In HGF, *BCL2*, *BAX*, and *CASP8* expression appeared to increase in a dose-dependent manner; however, only *BAX* and *CASP8* expression differed significantly in a dose-dependent manner ($p < 0.017$).

4. Discussion

Dental caries is one of the most common infectious diseases in humans and is treated by removing necrotic dental tissue and replacing it with restorative material. Because this restorative material will be in contact with dental and surrounding tissues for a long time, determining the biocompatibility of dental materials is of great importance. To date, a number of new dental materials have been developed, and their biocompatibility has been evaluated using either *in vivo* or *in vitro* tests [20–22]. However, new biological problems have arisen with the use of novel dental materials. One of the problems is health-related issues about the intraoral release of BPA molecules from resin-based dental materials [5]. In this regard, this study assessed the biocompatibility of newly developed 3D-printed resin materials in terms of BPA release.

The results of this study confirmed that BPA release was not detected in the 3D-printed resin but was detected in the conventional composite resin. Thus, the null hypothesis that there is no difference between BPA levels released from commercially available 3D-printed resin materials and composite resins was rejected. This finding corresponded to those of previous studies [23, 24]. Conventional composite resins are mainly composed of Bis-GMA and other dimethacrylate monomers, fillers, and photoinitiators [25]. Resin monomers such as Bis-GMA may cause genotoxicity and cytotoxicity by modulating cytokine release and cell apoptosis/necrosis [25]. Bis-GMA-based materials are also used in 3D-printed resins [1]. One possible explanation can be the difference in the degree of conversion after curing between the 3D-printed and composite resins. Previous studies evaluating the degree of conversion of 3D-printed resins have shown that the degree of conversion of 3D-printed resin ranges from 60% to 100% [26, 27]. A similar study on composite resins showed that the degree of conversion generally ranged below 50% [28]. The difference in the degree of conversion between the 3D-printed resins and composite resins appears to be due to the curing method and the number of curing cycles. Stereolithography (SLA) and digital light processing (DLP) are types of 3D printing widely used in dentistry. SLA and DLP printing are

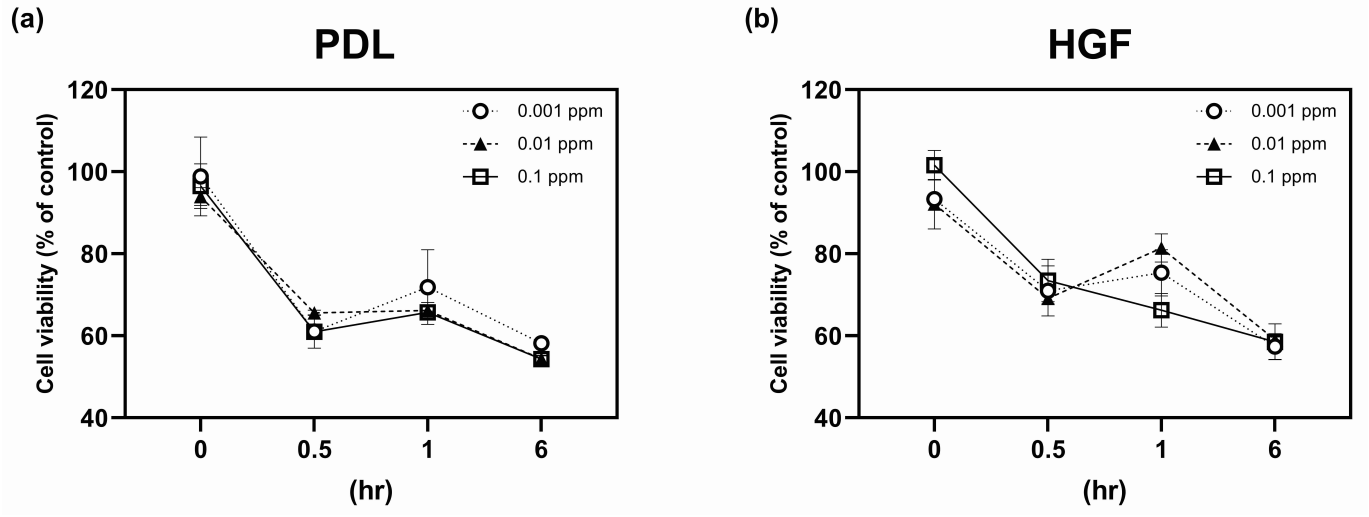

FIGURE 3. Cell toxicity test by CCK-8 assay. (a) Human periodontal ligament cell, (b) Human gingival fibroblast. PDL: periodontal ligament; HGF: human gingival fibroblasts.

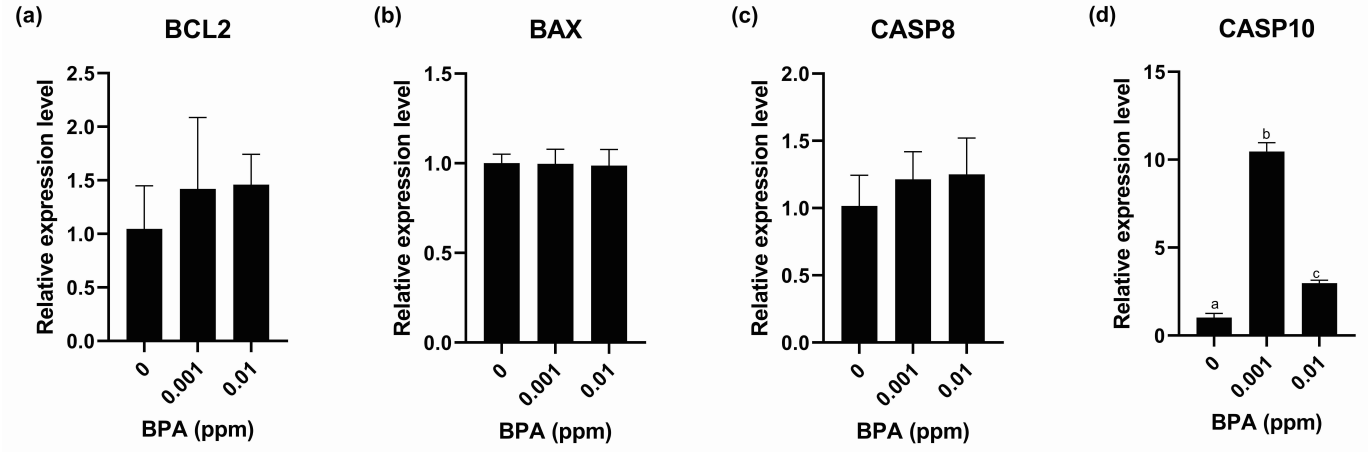

FIGURE 4. Apoptosis-related gene expression from PDL cells. (a) *B-cell lymphoma 2* (*BCL2*), (b) *BCL2 associated X* (*BAX*), (c) *Caspase8* (*CASP8*), (d) *Caspase10* (*CASP10*). The different upper case letters represent a statistically significant difference ($p < 0.017$).

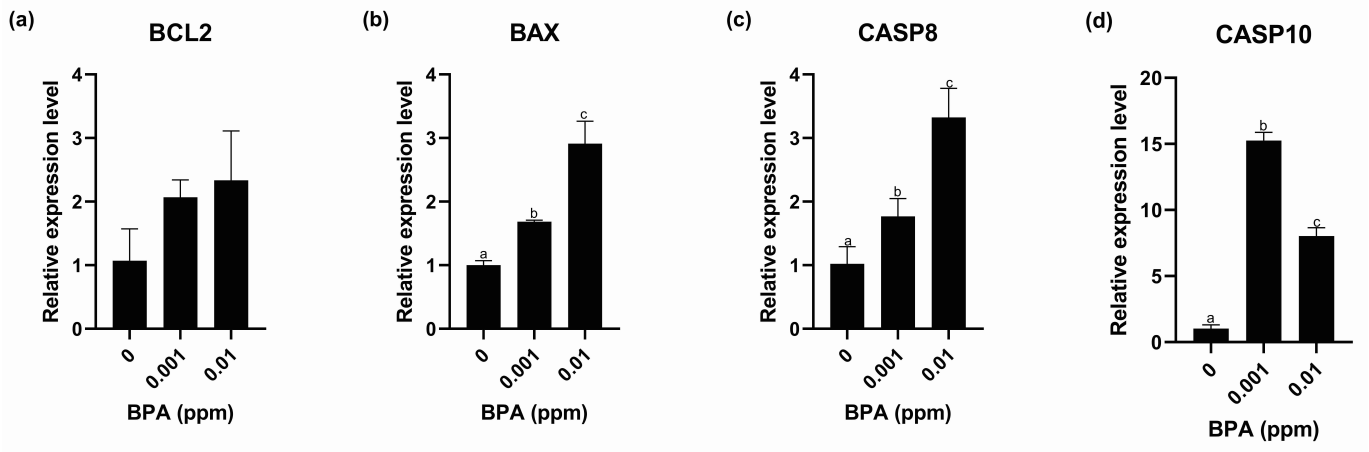

FIGURE 5. Apoptosis-related gene expression from HGF. (a) *B-cell lymphoma 2* (*BCL2*), (b) *BCL2 associated X* (*BAX*), (c) *Caspase8* (*CASP8*), (d) *Caspase10* (*CASP10*). The different upper case letters represent a statistically significant difference ($p < 0.017$).

additive manufacturing technologies that consist of building platforms, ultraviolet (UV) light sources, and resin tanks [29, 30]. They work on the same principle, but the printing speed is different owing to the difference in the light irradiation range. On the platform, a layer of liquid resin is selectively cured by UV light, and the liquid resin is converted to a solid resin. Solid resin is then used as the platform for the next liquid layer to be cured. This process allows the monomer resin to be polymerized layer by layer [29, 31]. Furthermore, 3D-printed resin dental prostheses generally undergo additional curing in separate curing machines by optimizing the temperature, according to the manufacturer's instructions. In contrast, conventional resins are usually cured after all the shapes are formed. Even with the incremental technique of composite resins, the number of polymerizations is inevitably lower than that of SLA. Another possible explanation may be the percentage volume difference in the Bis-GMA component between the materials. However, further studies are needed in this field.

In vitro assay was performed to further study BPA-related biological responses in HGF and PDL cells. Lower cell proliferation was observed in the BPA groups than in the control group in both HGF and PDL cells over time. Previous studies have confirmed that BPA exerts a cytotoxic effect on HGF [32]. This finding implies that the 3D-printed resins used in this study may be free of BPA-related cytotoxicity in the oral cavity.

HGF and PDL cells are the major cellular components of intraoral tissues and are essential for tissue regeneration and the preservation of tissue integrity [33]. However, the mechanisms by which BPA induces apoptosis in HGF and PDL cells by BPA have not yet been clearly elucidated. In this study, BPA exposure influenced apoptosis-related gene expression of both HGF and PDL cells. HGF exposed to 0.001 ppm BPA showed significantly higher apoptosis-related gene expression. BPA increased *BAX* and *CASP8* expression in a dose-dependent manner, consistent with previous studies [34, 35]. Another study confirmed that BPA induces apoptosis in HGF *via* caspases [32]. A previous study revealed that BPA induces apoptosis in a *BAX*/*CASP8*-dependent manner [34]. However, no significant differences were observed in *BLC2* expression in this study. Cell apoptosis can be induced through either the cell surface death receptor-mediated or mitochondria-mediated pathways. In the mitochondria-mediated pathway, the balance between *BCL2* and *BAX* is critical for cell survival or death [34]. Their actions on apoptosis are the opposite. *BLC2* inhibits apoptosis by preventing cytochrome c release, whereas *BAX* promotes apoptosis [35]. These mechanisms are required for BPA to initiate apoptosis of HGF and PDL cells.

This study has several limitations. First, due to analytic nature of this study, clinical situations of dental resin restoration, such as polishing procedure, were not completely simulated. Polishing after curing is an important step in ensuring the longevity of resin restorations as well as removing residual resin monomers from the tooth surface, as these monomers can be eluted into the oral cavity. Next, only short-term identification of BPA release was performed even if BPA release from the 3D-printed resins was not detected. Additionally, effects of BPA from 3D-printed resins on human oral cell apoptosis were not clearly identified because BPA released from 3D printed resins was not directly exposed to the cells. Finally, only gene expression levels were evaluated. Confirmation of the protein levels of BPA-related apoptosis is needed.

5. Conclusions

In conclusion, this study assessed BPA release from commercially available 3D-printed resin materials and confirmed that BPA was not released after curing. These findings suggest that 3D-printed resin materials are free from potential BPA-related risks. Regarding human cell apoptosis-related response to BPA, BPA exposure affected cell viability and apoptosis-related gene expression of HGF and PDL cells. This finding highlights the potential BPA-related risks to human oral cells.

AVAILABILITY OF DATA AND MATERIALS

The data are contained within this article (and supplementary material).

AUTHOR CONTRIBUTIONS

SWK, HL and OHN—designed the research study. YSJ, STR and OHN—performed the research. SWK, HL, YKC, KEL, H-SL, KHK, SKK and SCC—provided help and advice on the research. JSL—contributed the resources. YSJ and OHN—wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted following the Declaration of Helsinki. The study protocol was reviewed and approved by the Institutional Review Board of the Kyung Hee University Dental Hospital, Seoul, Korea (KH-DT22002).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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