Evaluation of the cytocompatibility of methacrylate resin-based root canal sealers with osteoblast-like cells

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This study compared the cytocompatibilities of three methacrylate resin-based root canal sealers [MetaSEAL Soft (MSS), Hybrid Root SEAL (HRS), and Superbond Sealer (SBS)] in either freshly mixed or set conditions using the Kusa A1 osteoblastic cell line. The three sealers and an epoxy resin-based sealer (AH Plus; AHP) were extracted in culture medium; cell growth and osteogenic properties were analyzed. Cell adhesion on set sealers was analyzed with scanning electron microscopy. The respective extents of cell growth were as follows in freshly mixed and set sealer extracts: SBS>MSS>AHP>HRS and SBS=AHP>MSS>HRS. Light irradiation of MSS and HRS increased the cell growth of set sealer extracts. Set SBS, MSS, and AHP did not alter expression of osteogenic genes or formation of mineralized nodules. Attached cells were observed only on SBS. In conclusion, the four sealers exhibited varying degrees of compatibility to osteoblasts; SBS and HRS were the most and least compatible, respectively.

Keywords: Cytocompatibility, Methacrylate resin-based root canal sealers, Osteoblasts, Scanning electron microscopy

INTRODUCTION

The primary objective of endodontic treatment is to eradicate intracanal pathogens and pathogenic substances. This is followed by hermetic sealing of the cleaned and shaped root canal space to prevent reinfection and entomb residual microorganisms¹. Regardless of the root-filling technique, sealers are necessary to establish hermetic sealing by filling the space between the dentinal wall and the obturating core material².

Available root canal sealers are currently composed of various formulations, including zinc oxide–eugenol, calcium hydroxide, silicone, calcium silicates, and several classes of resinous materials³. However, none of the products are considered ideal; in particular, “traditional” sealers (e.g., zinc oxide–eugenol-based materials) show no or low adhesiveness to dentinal walls⁴. Because of advancements in dentin bonding technology, several methacrylate resin-based sealers have been developed in attempts to establish simultaneous bonding of root canal sealers to root-filling materials and dentin⁵. Among these, Hybrid Root SEAL (HRS, also known as MetaSeal; Sun Medical, Moriyama, Japan) is a self-adhesive dual-curable sealer that contains 4-methacryloxyethyl trimellitate anhydride (4-META) as an adhesive monomer. MetaSEAL Soft (MSS; Sun Medical) is the next generation of Hybrid Root SEAL; it contains organic fillers to reduce the hardness of the set material, thereby improving removability if retreatment is necessary⁶. Superbond Sealer (SBS; Sun Medical) is a 4-META/methyl methacrylate-tri-n-butyl borane resin-

based sealer developed by modification of the Super-Bond resin cement.

Root canal sealers extruded from apices during root canal filling procedures may exert harmful effects on periapical tissue. In particular, freshly mixed sealers may induce periapical inflammation⁷; if they are in contact with osteoblasts on the alveolar bone surface, freshly mixed sealers may demonstrate cytotoxicity that negatively influences the repair and healing process. Furthermore, some harmful components released from set sealers may prolong periapical inflammation⁸. However, research has been inconclusive regarding the cytocompatibilities of methacrylate resin-based root canal sealers; notably, the methacrylate polymer is considered to exhibit negligible cytotoxicity in the set condition, but causes moderate cytotoxicity early in the setting process⁹. HRS exhibits less cytotoxicity¹⁰ and milder connective tissue reaction compared with a zinc oxide–eugenol sealer¹¹, but is more cytotoxic than AH Plus (AHP; Dentsply Sirona, Ballaigues, Switzerland), an epoxy resin-based sealer¹⁰. Compared with other methacrylate resin-based sealers (e.g., EndoREZ and RealSeal/Epiphany), HRS is reportedly either less cytotoxic¹² or induces more severe cytotoxicity¹³. SBS shows better cytocompatibility than methacrylate resin-based sealers^{14–16} and AHP¹⁶. Evidence concerning the biocompatibility of MSS is limited and conflicting; MSS has been reported to cause milder connective tissue reactions than AHP and a zinc oxide–eugenol sealer¹⁷, whereas it exhibits greater cytotoxicity than several other types of sealers¹⁷.

This study aimed to compare the cytocompatibility of MSS, HRS, and SBS in either freshly mixed or set conditions using the Kusa-A1 osteoblastic cell line. The

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null hypothesis was that there would be no difference in cytocompatibility among the three methacrylate resin-based sealers.

MATERIALS AND METHODS

Cell culture and sample preparations

Kusa-A1 cells^{18,19} (an osteoblastic cell line derived from mouse osteogenic mesenchymal progenitor cells; Cell No: RCB2081; Cell Engineering Division, RIKEN BioResource Research Center, Ibaraki, Japan) were cultured in alpha-modified minimum essential medium (α -MEM, Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in 5% CO₂. Culture medium was changed at 3-day intervals.

Preparation of sealer extracts

Three methacrylate resin-based sealers (*i.e.*, MSS, HRS, and SBS) and an epoxy resin-based sealer (AHP) were used in this study (Table 1). The sealers were mixed aseptically in accordance with the manufacturers' instructions and placed in sterile cylindrical plastic rings ($\varnothing$ 7 mm and height 3 mm; 250 mg per ring). They were immersed in 3 mL of α -MEM immediately after filling in the rings (*i.e.*, fresh samples) or following storage in a CO₂ incubator (5% CO₂ and 95% air) at 37°C for 24 h to allow complete setting. The prepared samples were then shaken in 3 mL of α -MEM at room temperature for 24 h (*i.e.*, set groups without irradiation). Light irradiation was performed immediately after the injection of MSS and HRS into sterile cylindrical plastic rings (*i.e.*, set samples). Light-irradiated samples of MSS and HRS [referred to as MSS(+) and HRS(+)] were prepared by irradiating the sealers filled in the ring with an LED light curing unit (Blue Lex, Yoshida, Tokyo, Japan; 1,400 mW/cm², 20 s); they were then immersed in α -MEM as described above. The media with sealers were shaken for 24 h at room temperature, sterilized with a syringe filter

(pore size 0.45 μ m, Sartorius, Göttingen, Germany), and used as sealer extracts for further experiments.

Cell viability assay

Kusa-A1 cells (3×10^3 cells/well) seeded in 96-well plates were cultured with 100 μ L α -MEM for 24 h, then cultured with sealer extracts for an additional 48 h. Cells cultured with α -MEM were used as control samples. Vital cell numbers were measured using the Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Kumamoto, Japan). Briefly, 10 μ L of CCK8 solution was added to the culture medium and the absorbance at 450 nm was measured with a spectrophotometer (TECAN Sunrise, Männedorf, Switzerland), following 1 h of incubation at 37°C.

Osteoblastic marker expression

Kusa-A1 cells (5×10^4 cells/well) were seeded in 24-well dishes and cultured with eightfold diluted set samples (MSS without light irradiation, HRS without light irradiation, SBS, and AHP) or α -MEM (control) for 72 h. Total RNA was extracted using an ultra-thin polymer membrane (QuickGene, Kurabo, Osaka, Japan), and cDNA was synthesized (PrimeScript RT reagent Kit: Perfect Real Time, Takara Bio, Shiga, Japan) from extracted RNA (150 ng). Real-time polymerase chain reaction was performed with Taq polymerase mixture including SYBR Green (GoTaq qPCR Master Mix, Promega, Madison, WI, USA), synthesized cDNA, and specific primers for osteoblasts using a real-time thermal cycler (CFX96, Bio-Rad, Hercules, CA, USA). PCR reactions were performed using 2-step cycling profiles (40 cycles) with 15 s denaturation (95°C) and 60 s annealing (60°C) per cycle following 2 min denaturation (95°C). Beta-actin was used as an internal control. The primer sequences were as follows: alkaline phosphatase (Alp; accession number NM_007431), anti-sense: 5'-GGAATGTAGTTCTGCTCATGGAC-3' and sense: 5'-GATTACGCTCACAACAACACTACCAG-3'; integrin

Table 1 Root canal sealers used in this study

Material	Manufacturer	Lot No.	Composition
MetaSEAL Soft	Sun Medical, Moriyama, Japan	Liquid: TW1 Powder: VF 1	Liquid: 4-META, HEMA, di-methacrylates, photoinitiator, water Powder: bismuth carbonate oxide, organic filler, aromatic amine
Hybrid Root SEAL	Sun Medical	Liquid: VG 11 Powder: TX 12	Liquid: 4-META, HEMA, di-methacrylates, photoinitiator Powder: zirconium oxide, amorphous silica, aromatic amine
Superbond Sealer	Sun Medical	Liquid: TE 2 Powder: TM 1 Catalyst: SW 33	Liquid: 4-META, MMA Powder: zirconium oxide (80%/w), PMMA (20%/w) Catalyst: TBB-O
AH Plus	Dentsply Sirona, Ballaigues, Switzerland	1911001017	Paste A: bisphenol A epoxy resin, bisphenol F epoxy resin, calcium tungstate, zirconium oxide, silica, iron oxide pigments Paste B: dibenzylidiamine, aminoadamantane, tricyclodecane-diamine, calcium tungstate, zirconium oxide, silicone oil, silica

4-META: 4-methacryloxyethyl trimellitate anhydride, HEMA: 2-hydroxyethyl methacrylate, MMA: methyl methacrylate, PMMA: polymethyl methacrylate, TBB-O: partially oxidized tri-n-butylborane

binding sialoprotein (Ibsp; accession number NM_008318), anti-sense: 5'-AGTAATAATTCTGACCCTCGTAGCC-3' and sense: 5'-TATGAAGTCTATGACAACGAGAACG-3'; β -actin (accession number NM_007393), anti-sense: 5'-GTAAAGACCTCTATGCCAACACAGT-3' and sense: 5'-AATGATCTTGATCTTCATGGTGCTA-3'.

Mineralized nodule formation

Kusa-A1 cells (2×10^4 cells/well) were seeded in 48-well plates and cultured in α -MEM containing 10% fetal bovine serum. After 24 h of culture, the medium was changed to an osteoinduction medium containing L-ascorbic acid (0.2 mM; Wako Pure Chemical Industries) and β -glycerophosphate (5.0 mM; Sigma Aldrich, St. Louis, MO, USA), with or without eightfold diluted sealer extracts. Mineralized nodules were stained with alizarin red S (Wako Pure Chemical Industries) after 10 days of culture. The mineralized nodule area was measured with ImageJ2 software (<https://imagej.net/ImageJ2>).

Scanning electron microscopy

Set sealers filled in the ring were rinsed with phosphate-buffered saline and placed in 48-well plates; Kusa-A1 cells (2×10^4 cells/well) were then seeded and cultured on the samples for 3 days. Following fixation with 2.5% glutaraldehyde (Wako Pure Chemical Industries), the specimens were dehydrated using a graded ethanol series, dried in a critical-point dryer (HCP 2, Hitachi, Tokyo, Japan), sputter-coated with platinum using an ion-coater (E102, Hitachi), and examined with a scanning electron microscope (Hitachi S-4500) at an accelerating voltage of 5 kV.

Statistical analysis

Data regarding cell viability and osteoblastic marker expression were compared among groups using one-way analysis of variance and Tukey *post hoc* test. Data regarding mineralized nodule formation were compared among groups using one-way analysis of variance and Dunnett's *post hoc* test. All statistical analyses were performed using statistical software (GraphPad Prism v8.1, GraphPad, San Diego, CA, USA), and *p*-values < 0.05 were considered to be statistically significant.

RESULTS

Cell viability

Figure 1 shows the viabilities of Kusa-A1 cells after exposure to fresh samples. Cells exposed to MSS and MSS(+) exhibited significant reductions in viability, compared with the control, at dilutions up to 1/8 and 1/2, respectively ($p < 0.05$); they exhibited viabilities comparable with the control at dilutions of 1/32 and 1/8, respectively. Cells exposed to MSS(+) exhibited significantly greater viabilities than cells exposed to MSS in comparisons of extracts with the same dilution ($p < 0.05$). For HRS and HRS(+), cell viabilities were below the limit of detection. For cells exposed to SBS, the viable cell count was comparable with the control at 1/2 dilution, whereas it was significantly higher

than the control at dilutions of 1/8 and 1/32 ($p < 0.05$). Cells exposed to AHP exhibited significant reduction in viability, compared with the control, at dilutions up to 1/32 ($p < 0.05$).

Figure 2 shows the viabilities of Kusa-A1 cells after exposure to set samples. Cells exposed to MSS and MSS(+) exhibited significant reductions in viability, compared with the control, at dilutions up to 1/8 ($p < 0.05$); cells exposed to MSS(+) exhibited significantly greater

Fig. 1 Viability of Kusa-A1 cells cultured with diluted extracts of freshly-mixed sealers.

Cells were cultured with diluted extracts of fresh samples with or without light irradiation, and cell viability was measured at 72 h with CCK8. Dilution ratio is indicated as fractions. Different letters indicate significant differences between groups ($n=4$, $p < 0.05$). MSS, MetaSEAL Soft; HRS, Hybrid Root SEAL; SBS, Superbond Sealer; and AHP, AH Plus. (+), light-irradiated. ND, not detected.

Fig. 2 Viability of Kusa-A1 cells cultured with diluted extracts of set sealers.

Cells were cultured with diluted extracts of set samples with or without light irradiation, and cell viability was measured at 72 h with CCK8. Dilution ratio is indicated as fractions. Different letters indicate significant differences between groups ($n=4$, $p < 0.05$). MSS, MetaSEAL Soft; HRS, Hybrid Root SEAL; SBS, Superbond Sealer; and AHP, AH Plus. (+), light-irradiated. ND, not detected.

viabilities than MSS in comparisons of extracts with the same dilution ($p < 0.05$). Cells exposed to HRS and HRS(+) also showed significant reductions in viability, compared with the control ($p < 0.05$); cells exposed to MSS showed a significant reduction in viability, compared with MSS(+), in extracts with the same dilution ($p < 0.05$). Cells exposed to SBS and AHP did not show significant differences in viability, compared with the control ($p > 0.05$).

Osteogenic marker expression

As shown in Fig. 3, exposure to eightfold diluted set MSS, SBS, and AHP extracts induced the expression of *Alp* and *Ibsp* in Kusa-A1 cells at levels similar to the control, with no significant differences among test extracts ($p > 0.05$). No expression of *Alp* or *Ibsp* mRNA

Fig. 3 Osteogenic marker expression in Kusa-A1 cells cultured with diluted extracts of set sealers. Cells were cultured with 8-fold diluted extracts of set samples, and *Alp* and *Ibsp* mRNA expression was measured at 72 h by real-time PCR using specific primers. There were no significant differences among groups ($n=4$, $p > 0.05$). MSS, MetaSEAL Soft; HRS, Hybrid Root SEAL; SBS, Superbond Sealer; and AHP, AH Plus. Hybrid Root SEAL is not indicated in this graph because RNA extraction was not possible because of the limited number of vital cells.

was detected in Kusa-A1 cells cultured with eightfold diluted set HRS extracts.

Mineralized nodule formation

As shown in Fig. 4, exposure to all eightfold diluted set sealer extracts induced mineralized nodule formation in Kusa-A1 cells. Cells exposed to HRS exhibited

Fig. 4 Mineralized nodule formation in Kusa-A1 cells cultured with diluted extracts of set sealers. Cells were cultured in the osteoinducing medium with 8-fold diluted extracts of set samples for 10 days and mineralized nodules were stained with alizarin-red staining solution. Stained nodules were quantified by ImageJ2 software. An asterisk mark indicates significant difference compared with control ($n=4$, $p < 0.05$). Images above each bar in the graph show two representative alizarin-red-stained cultures of the corresponding group. MSS, MetaSEAL Soft; HRS, Hybrid Root SEAL; SBS, Superbond Sealer; and AHP, AH Plus.

Fig. 5 Scanning electron microscopic observation of Kusa-A1 cells attached on the surface of set sealers. Kusa-A1 cells were cultured on set MetaSEAL Soft (A), Hybrid Root SEAL (B), AH Plus (C), and Superbond Sealer (D, E) for 3 days. (E) Higher magnification view of the boxed area in D.

significantly less mineralized nodule formation than the control ($p < 0.05$).

Cell attachment

Survival and attachment to sealers was not observed among cells exposed to MSS, HRS, or AHP (Figs. 5A–C). In contrast, substantial populations of well-spread, attached cells with numerous filopodia or pseudopodia were observed upon exposure to SBS (Figs. 5D, E).

DISCUSSION

This study demonstrated that exposure to MSS or HRS significantly reduced the viability of Kusa-A1 cells. The toxic effects of MSS and HRS were also clearly observed in scanning electron microscopy analysis where Kusa-A1 cells did not remain adhered during exposure to MSS or HRS. Notably, cells exposed to SBS showed significantly greater viability than cells exposed to MSS and HRS; cells cultured on SBS exhibited a well-spread shape with numerous cytoplasmic processes that may be associated with cell adhesion. Thus, the null hypothesis that cytocompatibility would not differ among the three methacrylate resin-based sealers was rejected.

The cytotoxicities of resin-based materials are primarily attributable to residual monomers released from these sealers^{20,21}. Among the constituents of the presently examined sealers, 2-hydroxyethyl methacrylate (HEMA) contained in MSS and HRS may be associated with the lower cytocompatibilities of these sealers, because HEMA is able to penetrate into the cell wall and cause cellular damage at low concentrations^{22,23}. A high concentration of HEMA (4 mg/mL medium) inhibits intracellular tyrosine phosphorylation, which is an important signal transduction messenger, in L929 fibroblasts²⁴. Cell growth inhibition and cell cycle perturbation are also induced by HEMA in human gingival epithelial cells and dental pulp cells²⁵. Furthermore, depletion of glutathione (an index of oxidative stress) and production of reactive oxygen species are both induced by HEMA²⁵. Notably, dimethacrylates, such as bisphenol A glycerolate dimethacrylate, urethane-dimethacrylate, and triethylene glycol dimethacrylate, have been reported to show higher cytotoxicity to human gingival fibroblasts, compared with HEMA²⁶, suggesting that dimethacrylates are also associated with the lower cytocompatibilities of HRS and MSS. Triacrylate, which is contained only in HRS, may also be associated with the inferior cytocompatibility of HRS compared with MSS, because triacrylate-derived acrylic acid exhibits greater toxicity than methyl methacrylate²⁷.

Light irradiation improved the cytocompatibilities of MSS and HRS, supporting the notion that the cytotoxicities of light-cured resin-based materials are inversely proportional to the light-curing time, which enhances the rate of monomer conversion²⁸. Thus, the severe cytotoxicity of HRS suggests a lower monomer conversion rate in this material, even when it has been light-cured. From a clinical perspective, light curing of

dual-curable resin sealers primarily tends to polymerize the coronal portion for early establishment of a tight coronal seal²⁹; it presumably shows limited effects in the apical portion because of restricted access for the curing light.

In this study, SBS exhibited the most prominent cytocompatibility. 4-META, the main functional monomer of the three sealers examined, has been reported to show mid-range cytotoxicity among monomethacrylates with functional groups²⁷. However, 4-META appears to be well tolerated, because SBS did not interfere with Kusa-A1 cell viability. This finding is consistent with the results of an earlier report, in which 4-META showed lower cytotoxicity to human dental pulp cells³⁰. Methyl methacrylate, the major component of SBS, reportedly less cytotoxic than many other resin monomers^{27,31}. Tributyl borane, a polymerization initiator, may reduce the amounts of residual monomers and free radical synthesis after polymerization³². The present findings are also consistent with the results of an earlier study, in which a 4-META/methyl methacrylate-*n*-butyl borane resin was found to be minimally cytotoxic to osteoblastic cells except during the early curing stage³³. This study also revealed that fresh and diluted SBS promoted the cell growth. SBS is reported to release boron³⁴, which stimulates cell growth in low concentrations (0.1–0.5 mM) via activation of mitogen-activated protein kinase pathways³⁵. Moreover, boron at physiological concentration induces lymphocyte proliferation³⁶ probably through its antioxidant potential³⁷. Accordingly, boron released from SBS may be involved in the promoted cell growth induced by fresh and diluted SBS samples.

In this study, the cytotoxicities of three methacrylate resin-based sealers were compared with the cytotoxicity of AHP, which is one of the most widely evaluated sealers³⁸. Fresh AHP showed substantial cytotoxicity, which is consistent with the results of previous investigations; this might be due to the release of unpolymerized epoxy resin monomers^{39,40}. AHP is reportedly formaldehyde-free, which represents an improvement from its predecessor, AH26; however, AHP has been found to release a small amount of formaldehyde during the setting process³⁹, which might be responsible for the observed cytotoxicity of fresh AHP extracts. In contrast, set AHP showed minimal cytotoxicity to Kusa-A1 cells. Similarly, several earlier studies showed that set AHP was minimally cytotoxic^{14,39,40}, although one study showed that set AHP severely interfered with cell viability¹⁶.

mRNA expression levels of *Alp* and *Ibsp* in Kusa-A1 cells were not disturbed by exposure to eightfold diluted set sealer samples, with the exception of HRS. This agreed with the results of our cell growth experiments and with prior findings that HRS diluent induces downregulation of *Alp* and *Ibsp* mRNA expression in cementoblasts^{13,41}. Mineralized nodule formation was also significantly disturbed in cells exposed to eightfold diluted set HRS, although the formation was confirmed. To induce mineralized nodule formation, standard

culture medium was changed to osteoinduction medium containing sealer samples after detection of confluency. Thus, mineralized nodule formation was induced in the remaining viable cells, although further cell growth may have inhibited in the presence of HRS samples. Cells exposed to eightfold diluted set MSS showed slight inhibition of growth, but did not exhibit disturbed osteogenic gene expression or mineralized nodule formation, suggesting minimal harmful effects of MSS on the osteogenic potential of osteoblasts.

Scanning electron microscopy images of cell culture experiments revealed that attached cells were only observed upon exposure to SBS; Kusa-A1 cells cultured in the presence of SBS appeared to be elongated and possess multiple cytoplasmic extensions that projected from the cells to the surrounding surface (Fig. 5D, E). These results may be attributed to various factors such as chemical composition, particle size, surface topography, and sample bioactivity. No attached cells were found upon exposure to MSS, HRS, or AHP, although cells exposed to set AHP extract did not demonstrate significant reductions of viability (Fig. 2). Cells placed on the samples may have been directly affected by non-diluted toxic components released from the samples. Moreover, AHP diluents have been reported to disturb mRNA expression of collagen type I⁽⁴¹⁾, a major component of extracellular matrix that is essential for cell attachment⁽⁴²⁾. Therefore, disturbance of extracellular matrix synthesis (e.g., through disruption of collagen type I mRNA expression) may have occurred in Kusa-A1 cells cultured on set sealers except SBS, which may have led to the absence of cell attachment upon exposure to MSS, HRS, or AHP.

CONCLUSION

Our findings indicate that MSS, HRS, SBS, and AHP exhibited varying degrees of cytocompatibility with Kusa-A1 osteoblasts, such that SBS and HRS were the most and least compatible, respectively.

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