Cell viability of fine powders in hybrid resins and ceramic materials for CAD/CAM

Mariko NAKAI1, Koichi IMAI2,3 and Yoshiya HASHIMOTO1

1 Department of Biomaterials, Faculty of Dentistry, Osaka Dental University, 8-1 Kuzuhahanazono-cho, Hirakata, Osaka 573-1121, Japan
2 Department of Tissue Engineering, Faculty of Dentistry, Osaka Dental University, 8-1 Kuzuhahanazono-cho, Hirakata, Osaka 573-1121, Japan
3 Graduate School of Health Sciences, Osaka Dental University, 1-4-4 Makinohon-cho, Hirakata, Osaka, 573-1144, Japan

Corresponding author, Mariko NAKAI; E-mail: nakai-m@cc.osaka-dent.ac.jp

Resin blocks and ceramic blocks for CAD/CAM crowns were cut into powders and separated into three particle size groups. Oxidative stress and cell viability were measured in 3T3 and FRSK cells. The results of cytotoxicity tended to be slightly higher for resin than for ceramics. The values also increased as the particle size decreased in the powders. In addition, incorporation into cells was frequently observed under SEM, suggesting that the particle size of easily incorporated dust is different among cell types. Fluorescence-activated cell sorter (FACS) showed an increase in apoptosis and a decrease in cell viability in most of the sample groups compared to the control group. Hematoxylin and eosin staining of the cells showed deep staining of the nuclei in the sample groups. It was found that oxidative stress cell viability and apoptosis appeared differently depending on the size of the particles and the type of cells.

Keywords: CAD/CAM, Composite resin blocks ceramic blocks, Cell viability

INTRODUCTION

The use of resin and ceramic materials for CAD/CAM crowns has rapidly increased in Japan due to an increase in esthetic demand from patients, in metal prices, and in metal-free repair to deal with metal allergy1). The resistance to wear and discoloration of resin materials for CAD/CAM crowns are excellent compared with those of conventional dental composite resins since prostheses using these are prepared by cutting a hardening body with a high rate of polymerization. In addition, UDMA and TEGDMA are used for the resin matrix in many cases, so that almost no unpolymerized layer is present and the structure is a 3-dimensional network structure, leading to excellent strength2). On the other hand, ceramic materials have color and transparency similar to those of natural teeth and are superior in hardness, wear resistance, chemical stability, and biocompatibility. Discoloration is also small; however, the impact resistance is slightly inferior. Ceramic materials are classified into those using silica and those using aluminum and zirconium as the principal ingredient3). Moreover, the repair material may wear with time in the oral cavity after prosthesis attachment4). The presence of microparticles released from a nano-filler-containing dental crown repair material due to wear of the material has been reported in literature5).

There are various methods for biological safety evaluation of dental materials, and they are specified by the international standards ISO 1993, ISO 7405, and JIS T 6001. Regarding cytotoxicity tests, there are various experimental methods for in vitro testing. Biosafety tests of materials for CAD/CAM, such as the direct contact test and extraction test, are performed using a block, and it has been reported that both resin and ceramic materials may have almost no or only a slight influence on cells in the conventional cytotoxicity test6-8). In addition to focusing on the effects on epithelial cells in the case of adhesion to the body surface, it is also necessary to consider the effects on connective tissues. This is because fine particles may enter connective tissues when adhering to wounded skin or mucous membranes. However, there have been no reports on these in a form other than block, such as fine powder. A difference in cytotoxicity in nano-and submicron samples compared with that observed in the conventional cytotoxicity test has been reported in the past9-12). Furthermore, differences in cytotoxicity due to differences in particle shape and among the cell types tested have been reported13-16). The oral cavity tissue is composed of mesoderm-derived connective tissue and ectoderm-derived epithelial tissue, suggesting that the result may be different between connective tissue cells and epithelial cells, even though the material is the same.

We tried evaluation using 2 types of rodent-derived...
cells. Fine powders of resin and ceramic materials for CAD/CAM crowns were prepared by cutting and were exposed to the two types of cells. Phagocytosis by the cells was observed, and the influence of the particle size of fine powder on oxidative stress, cell viability, and apoptosis was investigated accordingly.

**MATERIALS AND METHODS**

**Materials**

Three types of resin blocks and two types of ceramic blocks for CAD/CAM crowns generally sold in Japan were used in this study (Table 1).

**Preparation and analysis of powder samples**

An autoclaved diamond disc for dental (Diamantscheibe Ultraflex, NTI-Kahla, Kahla, Germany) purpose was attached to a micro motor handpiece, and each block fixed to the sample table was cut into powder. The powder was collected on sterilized aluminum foil, washed sufficiently, and sterilized with EOG gas.

Since the composition of the contained monomer and detailed information on the content of the three types of resin material were not disclosed, whether there was a large difference among the three types was analyzed using Fourier transform infrared spectroscopy (FT-IR; IRAffinity-1S, Shimadzu, Kyoto, Japan) and the quest single reflection ATR accessory (Shimadzu). In addition, powder samples were observed using a scanning electron microscope (SEM; S-4800, Hitachi High-Tech, Tokyo, Japan). The particles in the field of view were counted repeatedly, and 420–500 particles were finally recorded. Furthermore, the particle size distribution was determined from the SEM images using the image analysis software Image J (National Institutes of Health, Bethesda, MA, USA).

**Cell culture**

Mouse-derived fibroblast-like cells, Balb/c 3T3 cells (Fig. 1a), and fetal rat skin-derived keratinocytes, FRSK cells (Fig. 1b), were used in this study. 3T3 cells were obtained from the Riken BioResource Research Center (Ibaraki, Japan), and FRSK cells were obtained from JCRB cell bank (Osaka, Japan). D-MEM (FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with fetal bovine serum Hyclone™ (USDA Tested, processed in USA, lot. AD16384277, Cytiva, Tokyo, Japan) was used at a volume ratio of 10%.

**Preparation of test solutions and exposure of cells**

Each powder sample was added to the assay medium at a concentration of 1 mg/mL. After strong stirring using 2

![Inverted phase contrast microscopy image of each cell cultured in DMEM supplemented with 10% FBS.](image)

*a: Balb c/3T3 cells, b: FRSK cells*
a desk-top vibrator, the powder was separated into three particle size groups (40 μm or larger, 5–40 μm, and 5 μm or smaller) using two types of cell strainers (40 and 5 μm), and a total of 15 test solutions were thus prepared. The numbers of 3T3 and FRSK cells were adjusted to 1.0×10^4 cells/mL, and each cell type was seeded in a 96-well-multiplate (AGC TECHNO GLASS, Shizuoka, Japan) using a multi-pipet and were subjected to static culture for 24 h in a CO2 incubator (37°C, 5% CO2, 95% air). After confirming the normal extension of each cell on the bottom surface of the well under an inverted phase-contrast microscope (Olympus, Tokyo, Japan), the culture fluid was exchanged with 100 μL/well of each test solution, followed by 3-day static culturing in the CO2 incubator as described above. For the control group, an assay medium containing no sample was added accordingly.

**Evaluation**

1. Measurement of oxidative stress by ROS
   Since peroxides and free radicals are produced in cells due to oxidative stress by reactive oxygen species, damaged proteins, lipids, and DNA, thus impairing organelles, oxidative stress was evaluated by ROS assay. The number of cells was adjusted to 1.0×10^5 cells/mL, and each cell type was seeded in a 96-well-multiplate and subjected to 24 h static culture in a CO2 incubator. The culture fluid was exchanged with 100 μL/well of each test solution. After 6 h of culture, the test solution was discarded, each well was washed with DMEM, and a highly sensitive DCFH-DA working solution of ROS Assay Kit (Dojindo Laboratories, Kumamoto, Japan) was aliquoted at 100 μL/well, followed by incubation for 30 min. After the supernatant was removed, the cells were washed twice with DMEM. D-PBS (−) (Nacalai Tesque, Kyoto, Japan) was added to each well, and the absorbance at the measurement wavelength (excitation: 490 nm, emission: 510 nm) was measured using an absorbtopiometer. This experiment was performed four times.

2. Measurement of cell viability
   The MTT method, which measures the viability of cultured cells by colorimetry, was used to determine enzyme activity by reducing MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan dye. In both cell types, the number of cells was adjusted to 1.0×10^5 cells/mL, and the cells were seeded in a 96-well-multiplate and subjected to 24 h static culture in a CO2 incubator. MTT (Dojindo Laboratories) was dissolved in D-PBS to reach a concentration of 0.5 mg/mL; this MTT solution was aliquoted at 100 μL/well in the 96-well-multiplate and cultured in a CO2 incubator. The test solution was discarded, the formazan dye was eluted with an acidic isopropanol solution, and the absorbance at 570 nm was measured using an absorbtopiometer. The calculated value of each sample was divided by the value of the control group and presented as a percentage. This experiment was performed four times.

3. Observation of phagocytosis of powder particles
   An autoclaved cover glass was broken and placed in a 35-mm cell culture dish. 3T3 cells and FRSK cells adjusted to 1.0×10^4 cells/mL were seeded in an individual dish and subjected to 24 h static culture in a CO2 incubator. The adhesion and extension of the cells on the glass were confirmed using an inverted phase-contrast microscope. After removal of the culture fluid, each test solution was added, and the cells were subjected to static culture for another 24 h. The test solution was discarded, the dish bottom surface was washed with D-PBS (−) warmed to 37°C, and the cells were fixed with 2% glutaraldehyde solution warmed to 37°C for 1 h. Furthermore, the cells were washed twice with D-PBS (−) and dehydrated with 50–100% ascending series of ethanol. The solution was replaced with isoamyl acetate solution, and the cells were dried on glass using a critical point dryer (HCP-1, Hitachi, Tokyo, Japan). The sample was fixed with carbon tape, and the morphology of the two cell types was observed by SEM.

4. Annexin V-FITC/PI-stained fluorescence-activated cell sorter (FACS)
   3T3 and FRSK cells were harvested by trypsin treatment, washed twice with D-PBS, and centrifuged at 1,500 rpm for 3 min. The supernatant was discarded, and the pellet was suspended in 10-fold diluted binding buffer at a density of 1.0×10^4 to 1.0×10^6 cells/mL. The sample solution (100 μL) was transferred to a culture tube and incubated with 5 μL of FITC-bound Annexin V (Nacalai Tesque) and 5 μL of PI (Nacalai Tesque) for 15 min at room temperature in the dark. To each sample tube, 400 μL of 10-fold diluted binding buffer was added and analyzed by FACS (FACS Verse, Becton Dickinson, Franklin Lakes, NJ, USA) using Cell Quest Research Software (Becton Dickinson).

5. Hematoxylin and eosin (HE) staining
   3T3 and FRSK cells were seeded on sterile cover glasses and incubated in a CO2 incubator for 24 h. The culture medium and test solution were exchanged and incubated for another 24 h. HE staining was performed according to the conventional methods. Samples were fixed with 4% paraformaldehyde (Nacalai Tesque) for 30 min. After rinsing with water, the samples were treated with Meyer hematoxylin (3000-2, Muto Pure Chemicals, Tokyo, Japan) for 20 min, fractionally washed with 1% hydrochloric acid alcohol, and washed with 1% eosin Y solution (Muto Pure Chemicals) for 10 min. They were subsequently treated with an ascending series of 60% alcohol and sealed with glycerin (Nacalai Tesque). An upright microscope was used for observation (Eclipse Ci POL, Nikon, Tokyo, Japan).

**Statistical analysis**

The mean and standard deviation of the acquired values were determined, and one-way analysis of variance, subset comparison (Scheffe’s F method), and multiple comparisons (Bonferroni method) were performed accordingly.
RESULTS

Analysis of powder samples
1. Analysis of resin materials for CAD/CAM crowns by FT-IR
The results of the monomer content analysis by FT-IR are shown in Fig. 2. The band shape (3,450–3,350 cm\(^{-1}\)) was assigned to the N-H group due to the presence of UDMA. The sharp peak at 1,750–1,650 cm\(^{-1}\) was due to the coupling of the stretching vibration of the C-O group with the amide peak of UDMA. The band shape (1,700 and 1,100 cm\(^{-1}\)) due to ester bonding was observed in TEGDMA. No significant difference was noted among the three types of resin materials in UDMA or TEGDMA in the powder cut out after polymerization.

2. Particle size distribution
No large difference was noted in the granularity distribution of the powder among the three types of resin materials and the two types of ceramic materials. The particle size of the powders was widely distributed from 10 µm to 300 µm or larger. Particles with a size of 50 µm or smaller accounted for 59–65% and 10 µm or smaller accounted for 14–24%. The rate of particles with sizes ranging from 10 to 50 µm was the highest (Fig. 3).

Measurement of oxidative stress by ROS
The results of the 3T3 cells are shown in Fig. 4a. The resin material was used as one group, and the ceramic material was used as another group for subset comparison. There was almost no difference of ROS between the resin and the ceramic materials. From the multiple comparison test, a difference was noted among the three particle sizes. The ROS value was small in the 40 µm or larger group; however, almost no difference was noted between the 5–40 µm and 5 µm or smaller groups. Almost no difference was noted among the three products of resin material; however, in the ceramic materials, a significant difference was noted in IE compared to that in VM. The results for FRSK cells are shown in Fig. 4b. Similarly, the resin material was used as one group, and ceramic material was used as another group for subset comparison. No significant differences were noted between the resin and ceramic materials. From the multiple comparison test, the influence of particle size was small compared to that of the 3T3 cells. No significant differences were noted among the products of the resin and ceramic materials.

Measurement of cell viability
The results for 3T3 cell viability are shown in Fig. 5a. As seen in the ROS test, from the subset comparison, the cell viability for resin materials was statistically significantly lower than that for ceramic materials. From multiple comparison tests with particle shape and material as design factors, among the three types of resin material, the cell viability was the lowest in SB, and it increased in the order of AR and KA. Cell viability tended to be low in the resin materials compared to that...
Fig. 4 Effects of resin and ceramic materials on cells by ROS assays was used to detect mitochondrial reactive oxygen species, especially superoxides. Cells exposed only to culture medium were used as negative control. A group set comparison of resin and ceramic materials was performed. a: 3T3 cells, b: FRSK cells

Fig. 5 Cell viability was determined by the MTT assay to identify the effects of resin and ceramic materials on cells. The group without sample was used as a negative control, and the cell viability was set at 100%. A group set comparison of resin and ceramic materials was performed. a: 3T3 cells, b: FRSK cells

in the two types of ceramic materials. There was no large difference between VM and IE. Cell viability was lower in the resin than in the ceramic materials, and the difference was significant. Among the particle sizes, cell viability tended to be high when the particle size was 40 μm or larger for both resin and ceramic materials. In the 5 μm or smaller group, cell viability tended to be low.

The FRSK cell viability results are shown in Fig. 5b. Likewise, in the subset comparison, the cell viability was statistically lower in resin material than in ceramic material. In a multiple comparison test in the same way as for 3T3 cells, in the resin materials, cell viability was the smallest in AR as observed in the 3T3 cells. In ceramic materials, the value was lower in VM than in IE. Among the particle sizes, a tendency of decrease with a decrease in the particle size observed in the 3T3 cells was noted only in the resin, KA. In the other products, particle size and cell viability varied widely among the products.

Observation of phagocytosis of fine powder particles
The SEM images are shown in Figs. 6a–f, 7a–f, 8a–f, 9a–f. Incorporation of multiple 5 μm or smaller particles into the 3T3 cells was confirmed in both resin and ceramic materials. On the other hand, in the FRSK cells, incorporation of multiple particles of the 5–40 μm group was frequently observed in both resin and ceramic materials.

Annexin V-FITC/PI FACS staining
The percentages of apoptosis, necrosis, and cell viability determined by FACS are shown in Fig. 10 and Tables 2, 3. Compared to the control group, the percentage of apoptosis increased in 3T3 cells with 5 μm or smaller KA, with 40 μm or larger SB, 40 μm or larger AR, and with 5 μm or smaller VB and IE. Necrosis increased in all groups except for <5 μm IE in 3T3 cells; in FRSK cells, it increased in all groups in KA, smaller than 5 μm of SB, 5–40 μm of AR, and with 5 μm or smaller IE, but it was lower than that in the control group in other groups. Cell viability decreased in 3T3 cells compared to that
Fig. 6  a–f: SEM images of 3T3 cells with the resin particles incorporated. The white arrows indicate the unevenness of the cytoplasm due to the incorporation of resin particles smaller than 5 μm into the cell. a, b: 40 μm or larger; c, d: 5–40 μm; e, f: 5 μm or smaller.

Fig. 7  a–f: SEM images of 3T3 cells with the ceramic particles incorporated. The white arrows indicate that ceramic particles of less than 5 μm are taken into the cell and these pressurize the nucleus. a, b: 40 μm or larger; c, d: 5–40 μm; e, f: 5 μm or smaller.

Fig. 8  a–f: SEM images of FRSK cells with particles incorporated. White arrows indicate the uptake of 5–40 μm resin particles into the cell and the deformation of the cell. a, b: 40 μm or larger; c, d: 5–40 μm; e, f: 5 μm or smaller.

Fig. 9  a–f: SEM images of FRSK cells with particles incorporated. The white arrows show the deformation of the nucleus due to the pressure of the 5–40 μm ceramic particles. a, b: 40 μm or larger; c, d: 5–40 μm; e, f: 5 μm or smaller.

in the no-addition group, except for those with an IE 5 μm or smaller; in FRSK cells, it decreased in all groups except for those with a VB 40 μm or larger.
Fig. 10 a–d: Induction of apoptosis and necrosis in 3T3 cells and FRSK cells cultures by different materials after 24 h. FACS analysis after staining with annexin V/PI. Three distinct cell distribution patterns are visible: normal viable cells (lower left quadrant); apoptotic cells (lower right quadrant); necrotic and/or ‘apoptotic necrotic’ cells (upper right quadrant). a: non-treated 3T3 cells (negative controls), b: 3T3 cells with the 5 μm or smaller SB particles, c: non-treated FRSK cells (negative controls), d: FRSK cells with the 5–40 μm AR particle, a–d show the diagrams of one representative experiment. b, d reveal an increase in the proportion of apoptotic and necrotic cells.

Table 2 Induction of apoptosis and necrosis in 3T3 cells cultured with different materials after 24 h. FACS analysis after staining with annexin V-FITC/PI. Percentage of viable, apoptotic, and apoptotic necrotic/necrotic cells after a 24 h treatment with materials

| Materials | Size       | Apoptosis ratio (%) |   |   |   |
|-----------|------------|---------------------|---|---|---|
|           | 40 μm or larger | 5–40 μm | 5 μm or smaller |
| KA        | 7.36±1.76  | 7.35±0.75          | 5.82±1.21          |
| SB        | 5.37±1.30  | 7.59±0.88          | 6.25±1.14          |
| AR        | 5.37±5.11  | 6.41±1.19          | 8.41±0.33          |
| VM        | 10.94±2.43 | 6.44±0.50          | 5.99±6.87          |
| IE        | 8.25±4.72  | 9.16±2.64          | 4.70±4.99          |
| Control   |            | 6.13±4.60          |   |   |   |

| Materials | Size       | Necrosis ratio (%) |   |   |   |
|-----------|------------|--------------------|---|---|---|
|           | 40 μm or larger | 5–40 μm | 5 μm or smaller |
| KA        | 16.93±2.08  | 26.70±0.62         | 17.87±1.44         |
| SB        | 9.12±1.87   | 18.90±0.66         | 22.07±0.42         |
| AR        | 9.12±0.59   | 11.83±0.21         | 24.20±0.53         |
| VM        | 8.10±2.76   | 8.43±0.57          | 7.96±6.72          |
| IE        | 11.35±4.25  | 12.87±2.38         | 4.78±3.70          |
| Control   |            | 6.16±5.36          |   |   |   |

| Materials | Size       | Cell viability (%) |   |   |   |
|-----------|------------|--------------------|---|---|---|
|           | 40 μm or larger | 5–40 μm | 5 μm or smaller |
| KA        | 69.43±0.25  | 60.33±0.15         | 74.43±0.20         |
| SB        | 81.77±1.72  | 68.67±0.47         | 68.73±0.48         |
| AR        | 81.77±2.91  | 80.23±0.78         | 63.83±0.27         |
| VM        | 78.70±0.20  | 82.33±0.22         | 83.10±0.48         |
| IE        | 79.43±0.42  | 75.43±0.22         | 89.40±1.11         |
| Control   |            | 87.23±0.25         |   |   |   |

**HE staining**

HE staining images in the group without the sample and in the group with each sample are shown in Figs. 11, 12. The sample resulted in nuclear staining, nuclear
Table 3  Induction of apoptosis and necrosis in FRSK cells cultured with different materials after 24 h. FACS analysis after staining with annexin V-FITC/PI. Percentage of viable, apoptotic, and apoptotic necrotic/necrotic cells after a 24 h treatment with materials

| Materials | Apoptosis ratio (%) | Necrosis ratio (%) | Cell viability (%) |
|-----------|--------------------|--------------------|--------------------|
|           | 40 μm or larger    | 5–40 μm            | 5 μm or smaller    |
|           |                    |                    |                    |
| KA        | 22.53±5.54         | 22.47±1.03         | 18.37±0.37         |
| SB        | 26.90±3.96         | 12.21±20.19        | 18.73±3.44         |
| AR        | 26.27±4.96         | 34.37±8.06         | 16.80±2.59         |
| VM        | 10.20±0.39         | 17.37±0.76         | 14.10±1.85         |
| IE        | 26.70±5.37         | 25.10±1.48         | 16.07±1.37         |
| Control   | 9.01±6.21          |                    |                    |

Fig. 11  3T3 cells culture with ceramic and resin materials after 24 h.
HE staining of cells.

Fig. 12  FRSK cells culture with ceramic and resin materials after 24 h.
HE staining of cells.
expansion, and nuclear fragmentation, which are indicators of apoptosis. In addition, destroyed cells were also observed.

**DISCUSSION**

On comparison between the resin materials and ceramic materials, the measurement results of oxidative stress by ROS were found to be slightly higher in the resin than in ceramic materials, and the tendency was similar between the two cell types. However, a difference was noted in the results of cell viability between the connective tissue-derived 3T3 cells and epithelium-derived FRSK cells. In the 3T3 cells, there was a slight decrease in the resin materials compared to that in the ceramic materials. In FRSK cells, the cell viability was lower in the ceramic material, VM.

Regarding the influence of particle size, generally, the surface area per volume increases as the particle size decreases, with which various biological influences on the cells may increase. In the results of ROS-based measurement, the value was clearly higher in the 5 μm or smaller group than in the 5–40 μm and 40 μm or larger groups in the 3T3 cells in all products. In the FRSK cells, the variation was large among the particle size groups, as observed in the 3T3 cells. Incorporation of 5 μm or smaller particles into the 3T3 cells was frequently observed during the observation of phagocytosis of fine powder particles by SEM. Similarly, the incorporation of 5–40-μm particles was frequently observed in FRSK cells. From the MTT and ROS tests, it was predicted that the sample caused oxidative stress and cell death.

Necrosis occurs when cells are exposed to the materials. This occurs through the collapse of the cell membrane, after which its contents flow out, and proinflammatory factors such as digestive enzymes and cytokines have a serious effect on the surrounding cells. However, it has been reported that fine particles, such as nanomaterials, cause apoptosis, also known as programmed cell death. Apoptosis is caused by various factors, such as particle size, shape, and surface properties27-29). It is known that apoptosis is different from necrosis and it does not cause severe inflammation. Therefore, it is important to investigate the apoptosis and necrosis caused by fine dust grinding. The results of FACS suggested that there was more necrosis than apoptosis in 3T3 cells and increased apoptosis in FRSK cells. There are several possible causes of cell damage. In this experiment, it was difficult to observe a strong correlation between the results of ROS, cell viability, and cell phagocytosis; therefore, it is necessary to examine cell damage from multiple perspectives.

The three types of resin material tested in this study were resin/ceramic hybrids, and ceramics accounted for a large part of the composition, however it was possible that UDMA and TEGDMA contained in the resin materials, in which cytotoxicity decreased when these were polymerized, remained as a residual monomers with high cytotoxicity even though their amount was very small30). Moreover, although it could not be clarified which of the ceramic and resin materials were phagocytosed by cells at a higher rate, it is estimated that the angle of contact with water is larger in resin than in ceramic materials, and a large uptake of oily materials into cells has been noted. However, the involvement of multiple factors, such as particle size, wettability, hydrophobicity of the surface, and surface area, in phagocytosis has been reported. Therefore, no final judgment of whether phagocytosis had an influence could be made based on the results of this study26,30).

Regarding the mechanism of manifestation of toxicity of nano- and submicron-size materials, in addition to the direct action of chemical substances eluted from these materials on the cell membrane, the presence of a system actively incorporating these materials through phagocytosis by cells is known26,31). In the in vitro cytotoxicity test, although it depends on the test method, the cytotoxicity of materials insoluble in culture fluid is likely to be very low or lower than the detection limit22,31-33). Regarding the toxicity of insoluble particles, an association with particle size has been reported in literature. Matsuoka et al. exposed 11 types of 0.0024–92 μm polystyrene particles to Chinese hamster-derived CHL cells for 7 days and observed the cells by SEM. They observed that particles with a particle size of 0.92–4.45 μm were likely to be phagocytosed by cells, and the cytotoxicity and abnormal chromosome inducibility were high34). In our study, unlike polystyrene, which is spherical and has less surface roughness, surface unevenness was large on SEM observation and the cell type was slightly different, suggesting that the incorporated particle size was different.

The samples were vigorously stirred using a desktop vibrator; however, the possibility that the particles were not completely isolated cannot be ruled out. The isolation of nanoparticles is a major issue in nanomaterial research. The particle size of the samples in this study was larger than the submicron level, so that the particles separated through the cell strainers may have been mostly isolated by SEM observation. However, when the particle size is 5 μm or smaller, isolation by physical vibration alone may have been difficult, aggregates may have been slightly mixed, and this may have been the cause of the absence of a clear correlation between the particle size and the results of the MTT method.

Powders of resin materials and ceramic materials for commercial CAD/CAM crowns were prepared by grinding the material in a block shape using a diamond disc for dental use. However, mixing with the ingredients of the diamond disc, that is, particles of the diamond wheel and plating materials, such as nickel, needs to be considered35). In this study, a new diamond disc was used, which was prepared in an attempt to avoid contamination with foreign substances as much as possible, and the samples were sufficiently washed with running water. The ingredients of grinding/abrasive materials, such as discs and points, may be mixed in the dust in clinical practice.

In actual dental practice, in addition to the materials used in this study, complex particles, such as metals,
The influence of dust produced during dental practice on health is a concern. There have been many reports on the shape, character, and influence on the body of dust. Regarding the specific gravity of resin materials and ceramic materials, since the specific gravity of ceramic materials is higher than that of resin materials, resin materials may be more likely to stay in the air since their specific gravity is low and the particle size is small, predicting that materials staying in the air longer are more frequently exposed to the body.

In this study, the cytotoxicity level of resin materials with a small particle size increased slightly. It was thus clarified that it is necessary to pay close attention when fine powder of resin materials is produced in dental practice.

CONCLUSIONS

Ground fragments of three types of resin materials and two types of ceramic materials for CAD/CAM crowns were separated into three particle sizes (40 μm or larger, 5–40 μm, 5 μm or smaller), and oxidative stress by ROS, cell viability, and FACS of 3T3 cells and FRSK cells exposed to the particles were measured accordingly. The influence of the resin materials on the cells tended to be slightly larger than that of the ceramic materials, whereas the relationship with the particle size was different between the two cell types. In addition, phagocytosis of powder particles was observed by SEM, and incorporation of particles smaller than 5 μm was confirmed in 3T3 cells. In FRSK cells, incorporation of 5–40-μm particles into the cells was frequently observed, suggesting that the particle size of easily incorporated dust is different depending on the cell type. In addition, HE staining showed histological images of nuclear and cellular damage caused by the addition of the sample. In this study, the effects of CAD/CAM materials in the form of fine powder on cells were examined using basic data on biological safety, and it was found that cellular damage appeared differently depending on the size of the particles and the type of cells.

ACKNOWLEDGMENTS

The author would like to thank the members of the Department of Biomaterials and Institute of Dental Research, Osaka Dental University. The author would like to grateful to Dr. Tsumano N, Dr. Akimoto H, and Ph.D. Iwasaki K for their technical support of tissue culture and FACS.

REFERENCES

1) Sannino G, Germano F, Arcuri L, Bigelli E, Arcuri C, Barlattani A. CEREC CAD/CAM chairside system. Oral Implantol (Rome) 2015; 3: 57-70.
2) Engler ML, Guth JF, Keul C, Erdelt K, Edelhoff D, Liebermann A. Residual monomer elution from different conventional and CAD/CAM dental polymers during artificial aging. Clin Oral Investig 2020; 24: 277-284.
3) Hynkova K, Voborna I, Linke B, Levin L. Compendium of current ceramic materials used for the CAD/CAM dentistry. Acta Stomatol Marisienis 2021; 4: 7-17.
4) Lauvahutanon S, Hidekazu T, Shiozawa M, Iwasaki N, Asakawa Y, Oki M, et al. Mechanical properties of composite resin blocks for CAD/CAM. Dent Mater J 2020; 36: 270-283.
5) Ludovicetti FS, Trindade FZ, Werner A, Kleverlaan CJ, Fonseca RG. Wear resistance and abrasiveness of CAD-CAM monolithic materials. J Prosthodont 2019; 120: 318.e1-318.e8.
6) Furtado de Mendonca A, Shahmoradi M, Gouvea CVD, de Souza GM, Ellakwa A. Microstructural and mechanical characterization of CAD/CAM materials for monolithic dental restorations. J Prosthodont 2019; 28: e587-594.
7) Sonmez N, Gultekin P, Turp V, Akgurong G, Sen D, Mijiritsky E. Evaluation of five CAD/CAM materials by microstructural characterization and mechanical tests: A comparative in vitro study. BMC Oral Health 2018; 18: 5.
8) Tamim H, Skjerven H, Ekfeldt A, Renold HJ. Clinical evaluation of CAD/CMetal-ceramic posterior crowns fabricated from intraoral digital impressions. Int J Prosthodont 2014; 27: 331-337.
9) Batson RR, Cooper LF, Duqum I, Mendonça G. Clinical outcomes of three different crown systems with CAD/CAM technology. J Prosthodont 2014; 112: 770-777.
10) Wang W, Li T, Luo X, Zhang K, Cao N, Liu K, et al. Cytotoxic effects of dental prosthetic grinding dust on RAW264.7 cells. Sci Rep 2020; 10: 14364.
11) Cokic SM, Ghosh M, Hoet P, Godderis M, Godderis L, Hoet P, et al. Should we be concerned about composite (nano-)dust? Dent Mater 2012; 28: 1162-1170.
12) Choudat D, Triem S, Weill B, Vicrey C, Ameille J, Brochard
P, et al. Respiratory symptoms, lung function, and pneumoconiosis among self employed dental technicians. Br J Ind Med 1993; 50: 443-449.

15) Zentöhöfer A, Rammelsberg P, Schmitt C, Ohlmann B. Wear of metal-free composite crowns after three years in service. Dent Mater J 2013; 32: 787-792.

16) Vanoorbeek S, Vandamme K, Lijnen I, Naert I. Single-tooth restorations: A 3-year clinical study. Int J Prosthodont 2010; 23: 223-231.

17) Yesil ZD, Alapati S, Johnston W, Seghi RR. Evaluation of the wear resistance of new nanocomposite resin restorative materials. J Prosth Dent 2008; 99: 435-443.

18) Rizzo-Gorrita M, Herrmann-Galindo C, Torres-Lagares D, Villaronga-Figallo MA, Gutierrez-Perez JL. Biocompatibility of polymer and ceramic CAD/CAM materials with human gingival fibroblasts (HGFs), Polymers (Basel) 2019; 11: 1446.

19) Campaner M, Takamiya AS, Bitencourt SB, Mazza LC, de Oliveira SHP, Shibayama R, et al. Cytotoxicity and inflammatory response of different types of provisional restorative materials. Arch Oral Biol 2020; 111: 104643.

20) Alamoush RA, Kushnerov E, Yates JM, Satterthwaite JD, Silikas N. Response of two gingival cell lines to CAD/CAM composite blocks. Dent Mater 2020; 36: 1214-1225.

21) Bella B Manshian, Uwe Himmelreich, Stefan J Soenen. Standard cellular testing conditions generate an exaggerated nanoparticle cytotoxicity profile. Chem Res Toxicol 2017; 30: 595-603.

22) Nativo P, Prior IA, Brust M. Uptake and intracellular fate of surface-modified gold nanoparticles. ACS Nano 2008; 2: 1639-1644.

23) Mahmood M, Casciano DA, Mocan T, Iancu C, Xu Y, Mocan T, et al. Cytotoxicity and biological effects of functionalized nanomaterials delivered to various cell lines. J Appl Toxicol 2010; 30: 74-83.

24) Kroll A, Dierker C, Rommel C, Hahn D, Wohlleben W, Mitragotri S, et al. Polymer particle shape independently influences binding and internalization by macrophages. J Control Release 2010; 147: 408-412.

25) Champion JA, Mitragotri S. Role of target geometry in phagocytosis. Proc Natl Acad Sci U S A 2006; 103: 4930-4934.

26) Fröhlich E, Samberger C, Kuehnert T, Absenger M, Robleg E, Zimmer A, et al. Cytotoxicity of nanoparticles independent from oxidative stress. J Toxicol Sci 2009; 34: 363-375.

27) Manke A, Wang L, Rojanasakul Y. Mechanisms of nanoparticle-induced oxidative stress and toxicity. Biomed Res Int 2013; 2013: 942916.

28) Zhao T, Tan L, Zhu X, Huang W, Wang J. Size-dependent oxidative stress effect of nano/micro-scaled polystyrene on Karenia mikimotoi. Mar Pollut Bull 2020; 154: 111074.

29) Yin H, Too HP, Chow GM. The effects of particle size and surface coating on the cytotoxicity of nickel ferrite. Biomaterials 2005; 26: 5819-5828.

30) Hattori K, Nakadate K, Morii A, Noguchi T, Ogasawara Y, Ishii K. Exposure to nano-size titanium dioxide causes oxidative damages in human mesothelial cells: The crystal form rather than size of particle contributes to cytotoxicity. Biochem Biophys Res Commun 2017; 492: 218-223.

31) Hsu SY, Morris R, Cheng F. Signaling pathways regulated by silica nanoparticles. Molecules 2021; 26: 1398.

32) Watari F. Risk assessment and safety measures of nanomaterials. Fumiio W, editor. frontier-books; 2010. 96-103 p.

33) Matsuoka A, Onfelt A, Matsuda Y, Isama K, Sakoda H, Kato R, et al. Polydispersity induction by spherical size standard polystyrene particles in a Chinese hamster cell line CHL. Bull Natl Inst Heal Sci 2015; 2015: 29-36.

34) Orzech S, Czarnowska S, Stempik M, Klamrothova M, Klim A, et al. Polystyrene nanoparticle concentration and composition in a dental office and dental laboratory: A pilot study on the influence of working procedures. J Occup Environ Hyg 2018; 15: 441-447.

35) Nilsen BW, Jensen E, Örtengren U, Bang B, Michelsen VB. Airborne exposure to gaseous and particle-associated organic substances in resin-based dental materials during restorative procedures. Eur J Oral Sci 2019; 127: 425-434.

36) Breul S, Van Landuyt KL, Reich FX, Högg C, Hoet P, Godderis L, et al. Filtration efficiency of surgical and FFP3 masks against composite dust. Eur J Oral Sci 2020; 128: 233-240.

37) Liu MH, Chen CT, Chuang LC, Lin WM, Wan GH. Removal efficiency of central vacuum system and protective masks to suspended particles from dental treatment. PLoS One 2019; 14: e0225644.

38) Florez FLE, Thibodeau T, Oni T, Floyd E, Khajotia SS, Cai C. Size-resolved spatial distribution analysis of aerosols with or without the utilization of a novel aerosol containment device in dental settings. Phys Fluids (1994) 2021; 33: 085102.

39) Brune D, Beltesbrekke H. Levels of airborne particles resulting from handling alginate impression material. Scand J Dent Res 1980; 86: 206-210.

40) Brune D, Beltesbrekke H. Dust in dental laboratories. Part I: Measurement of particle size distributions. J Prosth Dent 1980; 44: 82-87.

41) Florez FLE, Thibodeau T, Oni T, Floyd E, Khajotia SS, Cai C. Size-resolved spatial distribution analysis of aerosols with or without the utilization of a novel aerosol containment device in dental settings. Phys Fluids (1994) 2021; 33: 085102.

42) Chen CT, Chuang LC, Lin WM, Wan GH. Removal efficiency of central vacuum system and protective masks to suspended particles from dental treatment. PLoS One 2019; 14: e0225644.

43) Florez FLE, Thibodeau T, Oni T, Floyd E, Khajotia SS, Cai C. Size-resolved spatial distribution analysis of aerosols with or without the utilization of a novel aerosol containment device in dental settings. Phys Fluids (1994) 2021; 33: 085102.

44) Brune D, Beltesbrekke H. Levels of airborne particles resulting from handling alginate impression material. Scand J Dent Res 1978; 86: 206-210.

45) Brune D, Beltesbrekke H. Dust in dental laboratories. Part I: Types and levels in specific operations. J Prosthet Dent 1980; 44: 687-692.

46) Bachetta R, Santo N, Valenti I, Maggioni D, Longhi M, Godderis L, et al. Polydispersity induction by spherical size standard polystyrene particles in a Chinese hamster cell line CHL. Bull Natl Inst Heal Sci 2015; 2015: 29-36.

47) Corazza PH, De Castro HL, Feitosa SA, Kimpara ET, Della Bon A. Influence of CAD-CAM diamond bur deterioration on surface roughness and maximum failure load of Y-TZP-based restorations. Am J Dent 2015; 28: 95-99.

48) Brune D, Beltesbrekke H, Strand G. Dust in dental laboratories. Part II: Measurement of particle size distributions. J Prosth Dent 1980; 44: 82-87.

49) Lang A, Ovsenik M, Verdenik I, Remškar M, Oblak Č. Nanoparticle concentrations and composition in a dental office and dental laboratory: A pilot study on the influence of working procedures. J Occup Environ Hyg 2018; 15: 441-447.