

Hydroxyapatite as a Root Canal System Filling Material: Cytotoxicity Testing

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ABSTRACT

Cytotoxicity testing, as a standard assay for toxicity of dental materials, is useful for initial biocompatibility evaluations. *In vitro* test of calcium hydroxyapatite (CHA) with established laboratory cell line showed multiple partitions of cells proving good biocompatibility properties of this new material. Further research should be directed towards simulation of *in vivo* conditions and animal experimentation to obtain sufficient data before its clinical application in humans.

1. INTRODUCTION

Direct contact between the root canal system filling material and the apical periodontal tissue, as well as communication through radicular accessory canals, apical ramifications or dentinal tubules, make the material biocompatibility crucial (1).

Biocompatibility of dental materials is evaluated on the basis of biological effect assessment by *in vitro* tests, animal experimentations and by clinical studies in humans. Cytotoxicity testing, as an initial step in testing biocompatibility, can include *in vitro* studies and the use of permanent cell lines and primary cell strains derived from living tissues (gingiva, PDL, pulp fibroblasts, mucosa) (2, 3, 4). Even though preclinical tests could not replace clinical studies, especially in assessment of dental materials, it has to be emphasized that simple, *in vitro* tests could reduce and partially replace more complicated and expensive animal tests or clinical studies without ethical problems (5, 6). Furthermore, cell cultures as biological systems for testing provide enhanced reproducibility and comparability of the results derived from different studies with the standardized methodology (6, 7, 8).

Research in endodontics has increased the potential application of different biomaterials (9, 10). Their synthetic nature eludes possible unwanted immune defence response and endodontic therapy compromise (11, 12). Furthermore, calcium phosphate biomaterials would be of great importance in the treatment of immature teeth as an apical plug material (13, 14). Even though they are easy to manipulate there is still a need for some improvements in their sealing properties and the researchers continue to search for an ideal composition of hydroxyapatite (15).

The present *in vitro* study tested cytotoxicity of three calcium phosphate biomaterials: hydroxyapatite synthesized in our laboratory (CHA), and two commercially available blends Apatite Root Sealer Type I and II (Sankin Industry, Japan).

2. MATERIALS AND METHODS

Cytotoxicity assays of three materials were performed according to the MEM Extraction Cytotoxicity Test, ISO/DIS 10993-5: Test for cytotoxicity: *in vitro* method (16,17). Negative control consisted of confluent monolayer cell strain grown on Minimum Essential Medium-Eagle

(MEM). Positive control consisted of confluent monolayer cell strain grown on MEM with addition of *neomycin sulphate* (*Sigma, USA*) in dose larger than permitted.

Human Diploid Lung WI-381 (ATCC Reference Strain CCL 75) monolayers in 25cm² tissue culture flasks, which represent normal diploid culture of embryonic human fibroblasts, were used in this study. This cell line was grown on a standard, Minimum Essential Medium-Eagle (MEM) (laboratory standard), supplemented with 5% foetal bovine serum. Cell proliferation was controlled by phase contrast optical microscopy (10x objective and a reduced light source). Greater than 80% confluent monolayer flasks (25cm²) of the appropriate cell type were selected.

Test materials used were: calcium hydroxyapatite (CHA) (derived from a precursor mixture of eggshell calcined at 900°C and (NH₄)₂HPO₄, by a hydrothermal precipitation method for 48h), Apatite Root Sealer-Type I, and Apatite Root Sealer-Type II, (Sankin Industry, Japan). Apatite Root Sealer Type I and II are based on α -tricalcium phosphate and hydroxyapatite, while Type II contains in addition 30% iodoform. The materials were mixed according to the manufacturers' recommendations.

The surface area of the sample was 60mm² and the thickness 0.5mm. The sample was cut into small pieces to optimize MEM contact and placed in a plastic 50 ml centrifuge tube with 20ml of 5% MEM. The extract of the test material was made at 37 ± 1° C for 24 hours. The tested materials and the controls were centrifuged at 1000 rpm for 10 minutes, to precipitate the solid substance and to prevent mechanical damage of cell cultures.

A total of three samples of each material were tested. Test materials, positive and negative controls were evaluated after observation periods of 24h, 48h, 96h and 7 days. Qualitative parameters were determined using a phase optical microscope fitted with a camera. They included examination of the cells microscopically to assess the changes in general morphology, vacuolization, detachment, cell lysis and membrane. The cultures were scored descriptively and numerically according to the ISO criteria (16). The sample would meet the requirements of the test if the cultures treated with the sample extract showed no greater than a mild reactivity (Grade 2) and if the suitability of the system was confirmed (i.e. positive and negative controls responded appropriately). Characteristic samples with selected area were prepared and observed by a scanning electron microscope (JEOL 6400F).

3. RESULTS

Results on the cell viability in CHA group after 24 and 48h showed biocompatibility identical to the negative control (Table 1). No cell lysis was observed in the samples at the evaluation periods (Fig. 1). In two other observation periods, no more than 20% of the cells were round, loosely attached and without intracytoplasmatic granules. Only occasional lysed cells were present in some samples after 96h and 7 days.

Table 1 Results of cytotoxicity assay for CHA

CHA sample	24h	48h	96h	7 th day
I	0	0	0	0
II	0	0	1	1
III	0	0	0	1

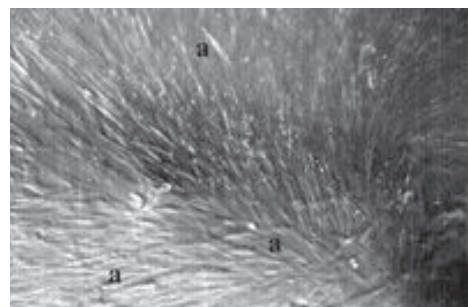


Fig. 1. No cell lysis observed with CHA after 96h; a-cell in partition

Scanning electron microscopy revealed normal general cell morphology of fibroblasts. Particles of CHA can be observed adjacent to the cells and on their surfaces (Fig. 2).

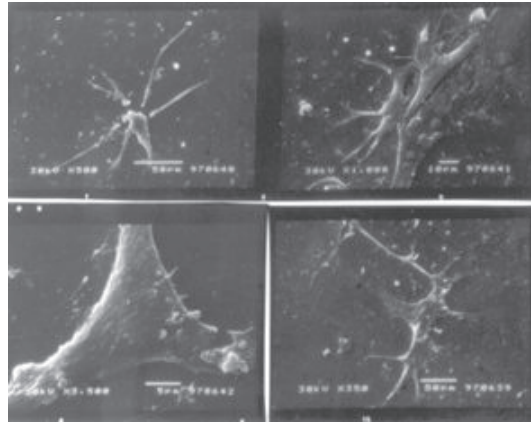


Fig. 2. SEM image of fibroblasts of normal morphology with CHA particles on their surface and surrounding them

In the first two evaluation periods, ARS Type I caused a slight reduction in cell culture viability up to 20%. For periods of 96h and 7 days, the toxic effect on the cells increased up to the level of mild cell reduction meaning that no more than 50% of the cells were round and devoid of intracytoplasmic granules (Table 2 and Fig. 3). Cell morphology and general fibroblast characteristics did not show any deviation from normal and multiple cell partitions, as observed microscopically.

Table 2 Results of cytotoxicity assay for ARS Type I

ARS I sample	24h	48h	96h	7 th day
I	1	1	2	2
II	0	1	1	2
III	0	1	1	1

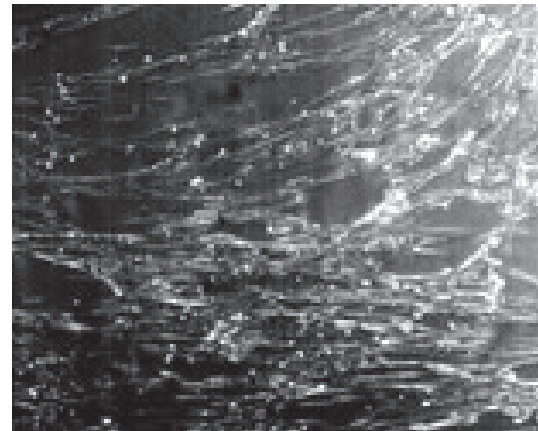


Fig. 3. Cell reduction with voids after 7 days with ARS Type I

ARS type II immediately after 24 h showed a mild reduction in cell viability, with no more than 50 % of cells being round and with voids between them. The same results were seen after 48h (Fig. 4), while cell lysis increased to moderate reduction after 96h and 7 days (Fig. 5). Nearly complete destruction of cell layers was seen in one sample after seven days (Table 3).

Table 3 Results of cytotoxicity assay for ARS Type II

ARS III sample	24h	48h	96h	7 th day
I	2	2	2	3
II	2	2	3	3
III	2	2	3	4

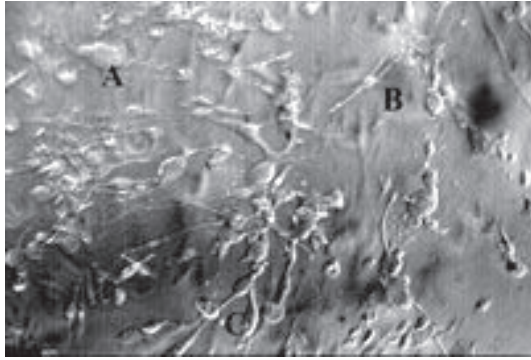


Fig 4. Cell culture of ARS Type II after 48 h, A, B, C-cells of changed morphology

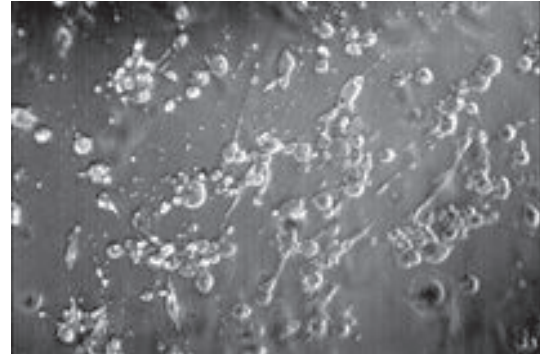


Fig 5. Cell culture of ARS Type II after 96h with dead cells and voids

Dead fibroblast cells with characteristic cellular swelling and vacuolization and complete absence of morphological characteristics were seen by the scanning electron microscope after 7 days in cultures of ARS Type II (Fig. 6).

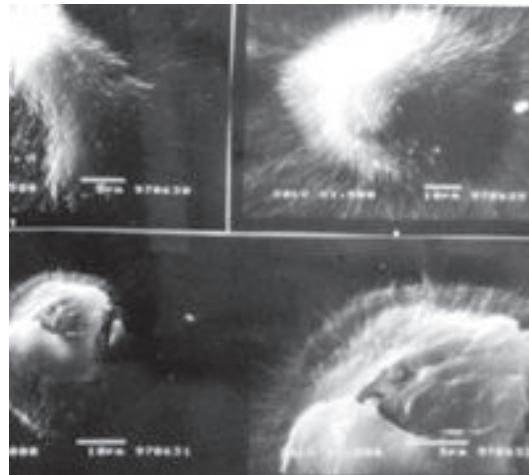


Fig 6. SEM image of ARS Type II samples, grade 4.

4. DISCUSSION

Cytotoxicity assays using established laboratory cell lines according to the ISO methodology proved to be simple and well defined, even though they did not have any specific features of pulp or PDL fibroblasts (18). They are used as a first approach to the standard assay for toxicity screening of dental filling materials and exhibit good reproducibility (6, 19).

Microscopic evaluation of CHA samples showed normal fibroblast morphology, while the degree of cell lysis was rather low. After four evaluation periods, in contact with the extract of the testing material, a monolayer of cells with normal morphology was formed. This layer looked very similar to the cells in contact with the extract of the negative control. SEM also confirmed complete preservation of fibroblast morphological and functional characteristics (Fig. 2). Our results obtained with CHA are in agreement with other studies reporting on a good biocompatibility of hydroxyapatite (20, 21, 22). Observed multiple partitions of cells agree very well with the generally accepted statement that biocompatibility means the absence of not only cytotoxic effect but also of positive effects in the sense of biofunctionality-promotion of biological responses. These results are very promising for further application of CHA.

The samples of ARS Type I after the evaluation periods met the requirements of the test since the cultures treated with the sample extracts showed quantitative reduction of cells under 50%. Preservation of fibroblast morphological characteristics as well as microscope evidence of cell multiplication during all evaluation periods confirmed functionally normal cultures. Reduction in the number of cells described as slight and mild according to ISO criteria was probably a result of the cumulative effect of soluble substances from the material. Chemical analysis of the eluates (extracts) would identify those substances, which demonstrate, even slightly, cytotoxic effect.

ARS Type I and CHA confirmed their biocompatibility in the present cytotoxicity study, while ARS Type II showed destruction of cell layers in most of the samples up to 70% after 96h and 7 days. Long periods of exposure of the cells to the ARS Type II materials resulted in increased cytotoxicity causing irreversible effects on the cellular metabolism and cell death or other serious negative effects on cellular function. Cell culture reduction ranging from slight to moderate was probably a consequence of the cumulative negative effect of harmful components that resulted from the lysis of fibroblasts in the closed system, such as laboratory flask with cell culture (Fig 6). The higher growth inhibition obtained in the cytotoxicity tests for ARS Type II was probably caused by the presence of iodoform. Differences in cytotoxicity values for ARS Type I and Type II arise probably from the toxic effect of iodoform since the basic composition of these sealers is identical, but there is insufficient evidence in the literature.

Interpretation of the results for the toxic effect on cell culture is very important because the tests do not give a biological assessment but a cytotoxic activity evaluation only. Abstract of the results could be wrongly interpreted, in which some medicaments are unsuitable for clinical practice even though their positive features and use in clinical practice are well known and irreplaceable (5).

Good biological reaction of cell strains on bioceramic materials presented by CHA was expected, but a cytotoxicity assay is necessary as the first step in acceptance of materials for clinical application techniques. Further research should be directed towards the use of primary cell lines with good simulation of *in vivo* conditions since results obtained from the cytotoxicity testing are not enough to gain adequate knowledge on the biocompatibility of a biomaterial (23).

5. CONCLUSION

On the basis of the results obtained in this study and their detailed analysis, it can be concluded that the test procedures recommended by ISO standards can easily be performed, are simple, highly reproducible, and clear presentation of the results is suitable for large scale and routine research. Cell cultures are useful for the assessment of dental materials, being fast and cost effective, and without ethical dilemma. Cytotoxicity testing revealed good biological acceptance (response) of samples of "pure" bioceramic materials (CHA and ARS Type I). Moderate cytotoxicity was reported on samples of ARS Type II (containing iodoform) but it has to be considered as approximate until correlation of the cytotoxicity effect rate with the results from application tests is established.

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