Cytotoxicity of gypsum-based biomaterial for direct pulp capping using stem cells from human exfoliated deciduous teeth

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Abstract

Aim: The aim of this study was to evaluate the cytotoxicity effects of experimental gypsum-based biomaterial prepared with various concentrations of chitosan (Gyp-CHT).

Materials and Methods: The study was performed using cell viability assay for mitochondrial dehydrogenase activity in stem cells from human exfoliated deciduous teeth (SHED), after 1, 2, and 3 days of exposure to the biomaterial extracts of varying concentrations. Differences in mean cell viability values were assessed by one-way analysis of variance, followed by Dunnett T3 post hoc test for multiple comparisons (P < 0.05).

Results: The cell viability to Gyp-CHT in low extract concentrations was statistically similar to that of the control and different from that of high extract concentrations. Gyp-5% CHT showed the highest percentage of cell viability with 110.92%, 108.56%, and 109.11%. The cell viability showed a tendency toward increment with low extract concentration and no constant effect of CHT on cell viability toward higher or lower.

Conclusions: Gyp-CHT biomaterial has no cytotoxic effects on the cultured SHED.

Keywords: Chitosan; cytotoxicity; direct pulp capping; gypsum; stem cells from human exfoliated deciduous teeth

INTRODUCTION

Direct pulp capping is defined as the treatment of a mechanical or traumatic vital pulp exposure by sealing the pulpal wound with a biomaterial placed directly on exposed pulp to facilitate the formation of reparative dentine and maintain the pulp vitality.[1] The ideal pulp capping material must be capable to stimulate the formation of reparative dentine, resist forces, sterile, radiopaque, bactericidal or bacteriostatic, provide bacterial seal, and maintain pulp vitality.[2]

The biocompatibility of pulp capping material is of great importance because the material will be in direct contact with pulp tissue for a long time, and to avoid pulp tissue irritation and degeneration. In reparative dentinogenesis, the initiated inflammation is followed by recruitment and differentiation of progenitor/stem cells at the exposure site to produce reparative dentine.[3] This would occur more predictably when the pulp is sealed with a biocompatible material that not only seals the pulp exposure to prevent egress of any bacteria but also to promote tissue repair, and aid injured structures reorganization.[4-5] The cytotoxicity of dental pulp capping materials to different cell lines has been widely studied.[6-9]

An experimental gypsum-based biomaterial has been investigated for use as a direct pulp capping material...
with high biological characteristics.[16] Chitosan (CHT) was incorporated into calcium sulfate (gypsum “Gyp”), both components have excellent biological properties particular to human tissue compatibility and regeneration process.

Gypsum is desirable in several respects of medical applications because it is biocompatible and nontoxic.[11] In addition, Gyp can also be used as a vehicle to deliver drugs to treat bone defects.[12,13] The calcium content of Gyp and its modulatory effect on gene expression profile of bone cells can support the formation of new bone.[14] Calcium ion, a significant component of gypsum, plays an important role in proliferation of dental pulp cells, Takita et al. reported that the proliferation of dental pulp cells was increased in a dose-dependent manner at higher calcium ion concentrations and that Mineral trioxide aggregate has a higher proliferation ability than Dycal due to its higher calcium ions concentration.[15]

Chitosan is a deacetylated derivative of chitin, found commonly in shells of crustaceans, marine, and fungi cell walls.[16] It is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine linked in a β (1–4) manner. Chitosan is biocompatible, nontoxic, and has antibacterial activity. Chitosan can prevent infection, accelerate wound healing, and enhance tissue growth.[17,18] The biocompatibility of chitosan is related to the structural similarities with glycosaminoglycans.[19] The development of Gyp-CHT biomaterial would obtain an alternative material with a lower cost and excellent biological properties for application in direct pulp capping.

The aim of the present study was to test the cytotoxicity of the experimental Gyp-CHT biomaterial with various concentrations of CHT to stem cells from human exfoliated deciduous teeth.

MATERIALS AND METHODS

Preparation of the biomaterial

PROTASAN UP CL 113 is well-characterized water-soluble component referred to as chitosan in this study (NovaMatrix, Norway; 75%-90% degree of deacetylation; molecular weight of 50,000–150,000 g/mol). Calcium sulfate dihydrate (Sigma-Aldrich, India) was heated at 110°C for 3 h in an electric oven (Universal Oven Memmert Life 600, Schwabach, Germany); as a result, calcium sulfate dihydrate was converted into a hemihydrate form (CaSO$_4$·½H$_2$O).[10] Water-soluble chitosan was used to increase the stability of chitosan in water and to decrease the acetic acid-induced cytotoxicity because nonwater-soluble chitosan was dissolved in an acetic acid solution.

CHT powder was added into distilled water to prepare a CHT solution with five different concentrations (10%, 5%, 2.5%, 1%, and 0% w/v CHT solutions). Gyp was mixed with the CHT solutions to prepare the biomaterial. The compositions of the Gyp-CHT biomaterial are shown in Table 1.

Cell culture preparation

SHED were purchased from AllCells, USA. The cells at passage eight were cultured in alpha minimum essential medium (α-MEM; Invitrogen, USA [GIBCO]) supplemented with 10% fetal bovine serum (Invitrogen, USA [GIBCO]) and 1% penicillin-streptomycin solution (Invitrogen, USA [GIBCO]). The cells were incubated in a humidified incubator with CO$_2$ at 37°C.[20]

Extract preparation

To evaluate the cytotoxicity in a dose-response relationship to the SHED, the in vitro elution test method (extract testing) was used. The biomaterial was mixed and placed into acrylic molds. After setting, the disks were retrieved, weighed, and exposed to ultraviolet light for 30 min (15 min for each side) in biological safety cabinet (Labconco, USA) to ensure sterility, and then transferred into sterile 15 ml centrifuge tubes.

Extracts of the test material were made by adding complete medium over each sample to obtain a concentration of 100 mg/ml according to ISO 10993-12[21] and 10993-5[22] the tubes were incubated for 3 days at 37°C. The extract was then drawn off and filtered by a sterile 0.2 μm filter (Minisart, Germany). After that, the extracts were serially diluted 1:1 with the complete medium into six concentrations of 50, 25, 12.5, 6.25, 3.13, and 1.65 mg/ml for each extract.

Cytotoxicity testing

Single cell suspensions of SHED were seeded in 96-well plates, in which 5 × 10$^3$ cells were transferred into each well as determined by hemocytometer counting in 100 μl of culture medium and incubated at 37°C and 5% CO$_2$ for 24 h.

The culture medium in the 96-well plates was replaced with 100 μl/well of aliquots of the test extracts. The wells without extract served as the control. The plates were then incubated at 37°C and 5% CO$_2$ for 3 days. Four wells were used for each single extract concentration.

MTS assay was performed to measure the cell viability. MTS is a tetrazolium salt-based colorimetric assay to detect the enzymes activity in mitochondria mostly reducing the MTS to formazan and gives a purple color.

On days 1, 2 and 3, 20 μl of CellTiter 96® AQueous One Solution Reagent was pipetted into each well of the 96-well plate containing the seeded cells in the biomaterial extract and incubated at 37°C for 4 h in 5% CO$_2$ humidified atmosphere.
The solution absorbance was then recorded by ELISA reader at test wavelength 490 nm. The cells cultured without biomaterial extracts were served as a control with cell viability of 100%. Then, the measurement of the cell viability was calculated using the following formula:

\[
\text{Cell viability} (\%) = \frac{\text{absorbance of samples}}{\text{absorbance of control}} \times 100
\]

**RESULTS**

Figure 1 shows the cell viability of the biomaterial using elusion method. The biomaterial with various CHT concentrations showed no significant difference with the control in low extract concentrations. Overall, lower extracts concentrations showed higher cell viability compared to the higher extracts concentrations. The cell viability showed a tendency toward increment with low extract concentration and no constant effect of CHT on cell viability toward higher or lower.

Gyp-5% CHT showed the highest percentage of cell viability with 110.92%, 108.56%, and 109.11%. At the highest extract concentration (100 mg/ml), Gyp-10% CHT showed significant less cell viability than Gyp-0% CHT on day 2 (\( P < 0.001 \)). Furthermore, Gyp-10% CHT and Gyp-5% CHT showed significant less cell viability than Gyp-0% CHT on day 3 (\( P < 0.05 \)). However, no statistical difference was found between Gyp-0% CHT and other biomaterials with CHT on days 1, 2, and 3. Within each of the lower extract concentrations (50, 25, 12.5, 6.25, 3.13, and 1.56 mg/ml), no significant difference (\( P > 0.05 \)) was found between Gyp-0% CHT and other biomaterials with CHT on days 1, 2, and 3, except Gyp-5% CHT with less cell viability than Gyp-0% CHT at 25 mg/ml on day 1.

In 100 mg/ml, biomaterial with high CHT concentrations showed less viability compared to biomaterial without/low CHT concentrations.

### Table 1: The compositions of the experimental biomaterial

| Biomaterial name | Liquid components (Chitosan concentration in 100 ml of distilled water) | Powder components (Calcium sulfate dihydrate [gypsum]) | Biomaterial ratio (Liquid/Gypsum) |
|------------------|------------------------------------------------------------------------|-------------------------------------------------------|----------------------------------|
| Gyp-0% CHT       | 0 g                                                                    | Gypsum                                               | 0.6 (100%)                       |
| Gyp-1% CHT       | 1 g                                                                    |                                                       | (ml/g)                           |
| Gyp-2.5% CHT     | 2.5 g                                                                  |                                                       |                                  |
| Gyp-5% CHT       | 5 g                                                                    |                                                       |                                  |
| Gyp-10% CHT      | 10 g                                                                   |                                                       |                                  |

Gyp-CHT: Gyp-CHT biomaterial

**DISCUSSION**

Viability tests are important steps in toxicity studies which determine the cellular response to a toxicant and give information on metabolic activities and cell death. Various test systems are used to evaluate the cytotoxicity of dental materials in mammalian cell cultures.[23] Replication assays test the cells ability to proliferate...
by evaluating the nucleotide analogues incorporation. Permeability assays test the cell membrane integrity by the release of radiolabeled chromium or by vital dyes inclusion or exclusion. Changes in cell surface or in the cellular cytoskeleton can also be detected morphologically. Finally, the cell’s ability to obtain the energy necessary for anabolic activities or the end products of such activities is evaluated by functional assays. MTS assay was employed in the present study to indicate the proliferation activity of cells. MTS is a tetrazolium salt-based colorimetric assay, used to detect the enzymes activity mostly in mitochondria which reduces the MTS to formazan giving a purple color.

Extracts of the test biomaterial offer the advantages of being prepared in a variety of concentrations to measure the dose-response relationship and determine the optimum concentration for the tested cells sensitivity. Extracts can also be sterilized by filtration easily, and the capability to evaluate the material effects on cells being in distant and in contact with them.

Primary cell strains derived from living tissues are essential to mimic the in vivo situation for specific sensitivity testing. SHED were employed as target cells in this case to mimic the dental pulp cells and to allow standardized and quick screening assessment to cytotoxic effects of dental materials.

In the present study, the general pattern exhibited a higher percentage of the cell viability with the decrease of biomaterial extract concentration. The biomaterial with high CHT concentrations exhibited less cell viability at high extract concentration while with low extract concentration, no significant difference between the biomaterials with/without CHT was observed. This indicates that possible cytotoxic effect of the biomaterial may affect the cells in contact to the application site in particular while the cells in distant location may have better viability or not affected.

Gyp-CHT showed good cytocompatibility in this in vitro study, and the biomaterial toxicity is still within the acceptable range for human use. The elicits of various dental materials has a cytotoxic response,[24] but it is not necessarily reflecting the long-term risk for the adverse effects. Often, the finding of clinical implication of a cell culture is difficult to interpret. The dental pulp tissue is generally more resistant to the toxicity of substances than cultured cells, because of repeated regeneration of new cells in vital pulp tissues.

**CONCLUSIONS**

We found that Gyp-CHT has no cytotoxic effects on cultured SHED. This observation suggests that Gyp-CHT is a promising candidate for use as a pulp capping material. Further in vivo studies should be undertaken to investigate its cytotoxic effects in clinical procedures.

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**Conflicts of interest**

There are no conflicts of interest.

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