Recebido em 18 mar. 2010. Aprovado em 12 jul. 2010

Cytotoxicity assessment of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) paste

Avaliação da citotoxicidade da pasta a base de caseína-fosfato de cálcio amorfo (CPP-ACP)

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Abstract

Introduction: Casein phosphopeptides (CPP) have been shown to be good carriers of calcium, phosphate, and hydroxide ions to promote enamel remineralization with applications in oral care products, professional dental products, and food products. Objectives: Evaluate the cytotoxicity of a casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) paste in rat fibroblasts. Materials and methods: Cytotoxicity was measured by the Trypan blue dye exclusion assay and the MTT assay. Results: Long term (1, 3, 5, and 7 days) and short term tests (0, 4, 8, and 12 hours) were performed with the Trypan blue dye exclusion assay. Data were analyzed with ANOVA and Kruskall Wallis tests. After one day, the cell viability of the CPP-ACP study group was between 80 and 100%. In the short term experiments, a reduction of cell viability in the study group was observed compared with the control group; yet viable cells were present over the evaluation period. With the MTT assay, a viability of 76.43% was observed in the study group, a significant difference from the control group (P = 0.003). Conclusion: CPP-ACP paste demonstrates low cytotoxicity in rat fibroblast culture.

Key words: Casein; Dental materials; Toxicity.

Resumo

Introdução: A caseína fosfopeptídea (CPP) tem demonstrado ser uma provedora de cálcio, fosfato e hidróxido, capaz de promover a remineralização do esmalte dentário, sendo utilizada em produtos de higiene oral, materiais dentários e alimentos. Objetivos: Avaliar a citotoxicidade da pasta a base de caseína (CPP-ACP) em fibroblastos de ratos. Materiais e métodos: A citotoxicidade foi avaliada pelos testes de exclusão azul de Tripan e MTT. Resultados: Testes de longo prazo (1, 3, 5 e 7 dias) e de curto prazo (0, 4, 8 e 12 horas) foram realizados com o teste azul de Tripan. Os dados foram analisados com os testes de ANOVA e Kruskall Wallis. Após um dia, a viabilidade celular do grupo de estudo CPP-ACP estava entre 80 e 100%. Nos experimentos de curto prazo, uma redução de viabilidade celular no grupo de estudo foi observada em relação ao grupo controle, mas as células viáveis foram observados durante todo o período de avaliação. Com o método MTT, foi observada no grupo de estudo a viabilidade de 76,43% f, com diferença significativa do grupo controle (P = 0,003). Conclusão: A pasta CPP-ACP demonstra baixa citotoxicidade em cultura de fibroblastos de ratos.

Descritores: Caseína; Materiais dentários; Toxicidade.

Introduction

The protective effects of milk and milk products against dental caries have been demonstrated in many animal studies[1]. In addition, casein phosphopeptides (CPP) have been shown to be good carriers of calcium, phosphate, and hydroxide (fluoride) ions to promote enamel remineralization with applications in oral care products, professional dental products, and food products[2].

The CPP are produced from tryptic digest of the milk protein casein by aggregation with calcium phosphate and purification by ultrafiltration. The CPP have a remarkable ability to stabilize calcium phosphate in solution and substantially increase the level of calcium phosphate in dental plaque[3], maintaining a state of supersaturation with respect to tooth enamel, reducing demineralization, and enhancing remineralization[4]. Casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP) were shown to have anticariogenic potential in laboratory, animal, and human *in situ* experiments[4-8]. The CPP-ACP interacts with fluoride ions to produce an additive anticariogenic effect through the formation of a stabilized amorphous calcium fluoride phosphate phase[3, 5].

Reynolds[9] compared the ability of CPP-ACP, and other forms of calcium, to be retained in supragingival plaque and remineralize enamel subsurface lesions *in situ* when delivered in a mouthrinse or sugar-free gum in randomized, double-blind trials. The results showed CPP-ACP were superior to other forms of calcium in remineralizing enamel subsurface lesions. In the same year, Mazzaoui[10] determined the effect of incorporating CPP-ACP into self-cured glass-ionomer cement (GIC). The release of CPP-ACP and fluoride from the CPP-ACP containing GIC was associated with enhanced protection of the adjacent dentin during acid challenge *in vitro*, suggesting 1.56%-CPP-ACP-containing GIC might be a superior restorative/base with improved anticariogenic potential. The CPP-ACP, unlike fluoride, can be added to sugar-containing foods; therefore, there is commercial potential as an additive to foods as well as to toothpastes and mouthwashes for the control of dental caries[2].

Biocompatibility can be defined as the ability of a material to perform with an appropriate host response in a specific application. Biocompatibility can be influenced by toxicity of the material. To evaluate if the material is biocompatible, *in vitro* tests and clinical studies may be performed[11]. The biocompatibility of materials used in dentistry is of considerable importance since it influences outcome and clinical application. An ideal dental material will not irritate living connective tissue. High biocompatibility renders a material useful in applications requiring contact with vital tissues.

Objective

The aim of this study was to evaluate the *in vitro* toxicity of a CPP-ACP paste in a continuous cell lineage of rat fibroblasts with high inhibition contact.

Materials and methods

Cell culture

NIH-3T3 fibroblasts (ATCC CRL 1658), obtained from the American Type Culture Collection (Rockville, MD, USA), were grown at 37 °C in Dulbecco's Modified Eagle Medium (DMEM, Sigma Chemical Company, St Louis MO, USA) supplemented with 10% fetal bovine serum (Cultilab Ltda, Campinas, SP, Brasil) and 1% antibiotic/antimycotic solution (Sigma Chemical Company, St Louis MO, USA) in a humid 5% CO_2 atmosphere. Cultures were supplied with fresh medium every other day.

Trypan blue dye exclusion assay

Cells (1 X 10^4) were plated on 60-mm diameter culture dishes as described previous-

ly[12]. Experimental groups were as follow: GI: control cultures receiving plain round coverslips, and GII: cultures receiving round coverslips coated with CPP-ACP paste. For short-term analysis, cells were counted after 0, 4, 8, 12, and 24 h and viability curves generated. For long-term analysis, cells were counted and respective growth curves plotted 1, 3, 5, and 7 days after seeding. Growth curves were generated as described elsewhere[12,13]. Briefly, cell counts were performed by counting the number of viable cells in a hemocytometer using the Trypan blue dye exclusion assay. Three dishes from each group were counted for each time period. The number of viable cells harvested from each Petri dish was obtained from the following equation: UC x D x 10^4/nSQ, where UC = unstained cell count (viable cells), D = dilution of the cell suspension, and nSQ = number of quadrants counted. The viability percentage of the cell population of each Petri dish was obtained by the following mathematical equation: UC/TC x 100, where UC = unstained cell count (viable cells) and TC = total cell count (stained plus unstained cells). Each data point corresponds to the mean ± SEM (standard error of the mean) of either cell count or percentage of cell viability from 3 dishes. The data obtained by Trypan blue exclusion were analyzed using the ANOVA and Kruskall-Wallis test. A *P* value of < 0.05 was defined as significant.

Culture medium conditioning

To obtain conditioned media (e.g. media containing substances dissolved or leached from the test materials), 50 mL centrifuge tubes containing the materials were filled with Dulbecco Modified Eagle Medium (DMEM, Sigma). Conditioning was carried out for 24 h at 37°C, using 0.2 g of each substance per mL of fresh medium, according to the American Society for Testing and Materials[14] and Cavalcanti et al[15]. Conditioned media was added to cell cultures and cell viability was measured 24 h later by analysis of cell mitochondrial activity.

MTT assay

This assay focuses on the capacity of mitocondrial dehydrogenase enzymes in living cells to convert yellow water-soluble tetrazolium salt 3- (4,5-dimethylthiazol-2-yl) –2,5-diphenyltetrazolium bromide (MTT; Sigma) into dark-blue formazana crystals. The water-insoluble product is stored in the cytoplasm of living test cells. The amount of formazana formed is directly proportional to the mitochondrial enzyme activity in a given cell line and is determined by optical density at 570 nm. A MTT reduction analysis kit (Vybrant MTT, Molecular Probes, Eugene, OR, USA) was used. Immediately after the assay procedure, the absorbance was read with a micro plate reader (Biotrak II, Biochrom Ltd, Eugendorf, Austria) using a 562 nm filter. The absorbance data was used to plot cell growth curves. The data obtained from the MTT method were analyzed using the t-Student test and a *P* value of < 0.05 was defined as significant.

Results

The number and percentage of viable cells in long term experiments by Trypan blue exclusion are presented in Figures 1 and 2, respectively. The number and percentage of viable cells in the short term experiments, according to the Trypan blue exclusion method, are shown in Figures 3 and 4, respectively. In the start point (first period measured) group II has always less cell viability than group I because group I (control) did not present any material. The presence of material in coverslips may already induce a semall initial toxicity, which does not mean that the material is totally toxic. Figure 5 shows the percentage of viable cells as determined by the MTT method.

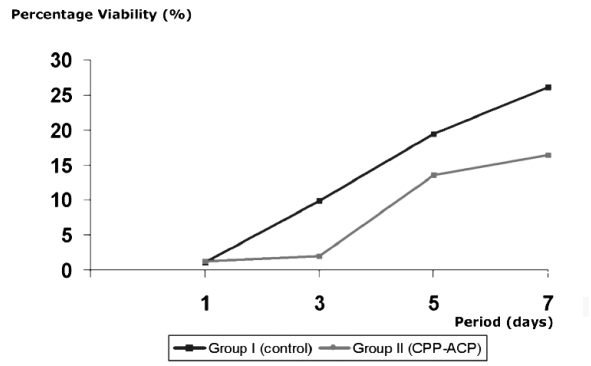

Figure 1: Number of viable cells in the long term experiment as determined by the Trypan blue dye exclusion assay

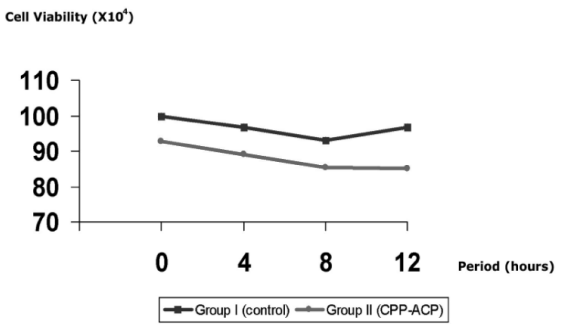

Figure 4: Percentage of viable cells in the short term experiment as determined by the Trypan blue dye exclusion assay

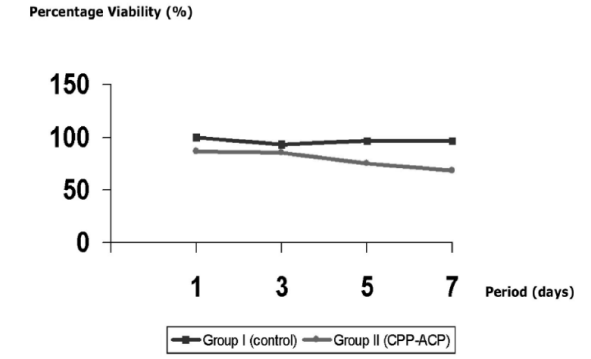

Figure 2: Percentage of viable cells in the long term experiment as determined by the Trypan blue dye exclusion assay

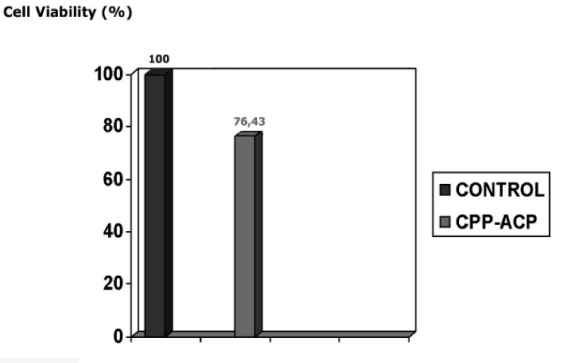

Figure 5: Percentage of viable cells as determined by the MTT method

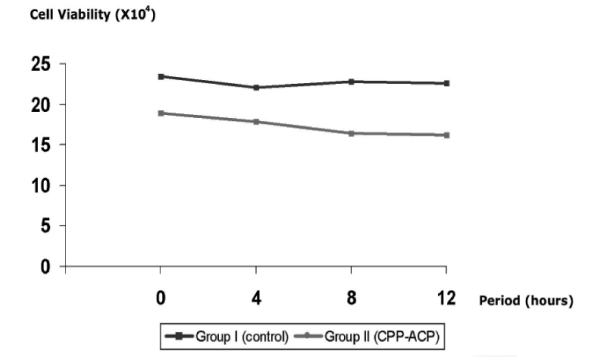

Figure 3: Number of viable cells in the short term experiment as determined by the Trypan blue dye exclusion assay

Discussion

Previous studies on casein phosphopeptide-amorphous calcium phosphate nanocomplexes focused on the process of demineralization and remineralization associated with the initiation of caries, as CPP-ACP had anticariogenic potential in laboratory, animal, and human *in situ* experiments[4-8]. According to the FDI[16] recommendations; initial, secondary, and application tests should be completed to determine the biological response of a material and the initial test should be performed *in vitro*. Measurements

of cytotoxicity and viability *in vitro* are specifically designed to assay viability or survival in response to a substance; thereby establishing a cheaper, more reproducible substitute for animal studies[17]. Although test results may not be directly correlated with *in vivo* results, a dental material which induces a cytotoxic reaction in cell culture will most likely also do so when in contact with oral tissue[18].

In the present study the biocompatibility of CPP-ACP was evaluated *in vitro* according to ISO 10993-Part 5[19] using two testing methods, the Trypan blue and MTT method. The MTT assay[20] is dependent on the intact activity of the mitochondrial enzyme, succinate dehydrogenase, which is impaired after exposure of cells to toxic surroundings. Briefly, the test involves the conversion of a tetrazolium salt 3- (4,5-dimethylthiazol-2-yl) –2,5-diphenyl to an insoluble formazan product, which can be quantified by spectrophotometry[21]. The trypan blue exclusion assay was used to evaluate the loss of cell-membrane integrity during cell death. Established cell lines have enhanced reproducibility of results and are recommended by the ISO for preliminary cytotoxicity screening; therefore, NIH 3T3 cells were used in this study.

The long term tests (Figures 1 and 2) showed there was no difference between the study group and the control after one day of contact with the CPP-ACP material. After that, the CPP-ACP group had significantly less cells than the control group, but a cell viability of greater than 70%. In the short term experiments (Figures 3 and 4), a reduction in the number of the viable cells was observed immediately after contact with the CPP-ACP paste in comparison with the cells of the control group. After 4 hours, a viability of 80 to 90% was observed. There was a reduction in the viability of the cells treated with CPP-ACP (P= 0.003) in the MTT assay (Figure 5), yet cell viability was 76.43%.

Although a reduction in the number of viable cells was observed, the CPP-ACP paste allowed cell viability greater than 70%. If the level of toxicity in relation to cells does not exceed the potential of healing, this level of cytotoxicity could be acceptable[22]. In conclusion, CPP-ACP paste demonstrate low cytotoxicity in rat fibroblast culture. As this level of toxicity is low and considering that the material is used topically, may conclude that its use is safe in destistry.

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