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In vitro evaluation of casein phosphopeptide-amorphous calcium phosphate as a potential tooth transport medium: viability and apoptosis in L929 fibroblasts

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Abstract – Casein phosphopeptides (CPP) are derived from casein, which accounts for 80% of the total protein in bovine milk .The purpose of this *in vitro* study was to evaluate the potential use of a CPP-amorphous calcium phosphate (CPP-ACP) preparation as a transport medium for avulsed teeth. L929 fibroblastic cell line was plated in 24-well culture plates. Following incubation, the cells were treated with 10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-12} dilutions of a water-based CPP-ACP paste (Tooth Mousse, GC Corp., Tokyo, Japan). Untreated cells served as controls. The L929 cells were counted at the 1st, 3rd and 7th days. Propidium iodide/acridine orange staining was used to assess apoptosis of treated cells and of the positive control. For each concentration (dilution), statistical analysis of cell survival within time was performed using two-way analysis of variance (ANOVA, $P = 0.05$). One way ANOVA and Tukey tests were applied to compare the effect of different concentrations on cell survival at each evaluation day ($P = 0.05$). Except for the $10^{-3}$and 10^{-4} dilutions, all groups demonstrated an increase in cell numbers at days 1 and 3, followed by a decrease at day 7. Irrespective of the increase or decrease in cell viability, time-dependent changes for each dilution group were significantly different. Cells in the $10^{-3}$and 10^{-4} dilution groups demonstrated a rapid apoptotic response. A relatively few number of apoptotic cells were observed in the 10^{-6} and 10^{-8} dilution groups, while no sign of apoptosis was evident in the 10^{-12} dilution group and control. These results suggest that when highly diluted, the tested CPP-ACP preparation may help preserve L929 cell viability in the short term without inducing apoptosis.

Avulsion injury is one of the most severe types of traumatic dental injuries, characterized by complete displacement of tooth from the alveolar socket. Following avulsion, periodontal ligament (PDL) tissues are injured, and the vessels and nerves of the pulp rupture at the apical foramen which, in turn, leads pulp tissue to undergo necrosis (1). The reported incidence of avulsion ranges from 1 to 16% of all traumatic injuries of the permanent dentition (2).

In case of avulsion, the main goal is to achieve success in replantation by avoiding or minimizing two possible complications: PDL damage and pulpal infection. Although it has been shown that the ideal treatment of avulsion injury is immediate replantation (1), this can rarely be achieved (3). PDL cells can be expected to survive a dry time of 15 min or less (4), but are unlikely to survive a dry time of greater than 60 min (5). Thus, teeth that have been kept dry for greater than 15 min, but less than 60 min still pose a treatment problem. At this point, a suitable transport media is extremely important to maintain the PDL cell viability and to help prevent the squealae of inflammatory resorption and replacement resorption.

Many studies have been carried out in the search of the most suitable storage media for avulsed teeth. The tested storage media include saliva, tap water, saline, milk, Hank's balanced salt solution (HBSS), Viaspan and cultured medium (6–8). Today, there is good evidence that supports the use of HBSS as the most suitable transport medium as it may help reduce replacement resorption by maintenance of a normal PDL (8, 9). If HBSS is not available, milk is reported to be the best alternative (10, 11).

The efficacy of milk in maintaining PDL cell viability can be attributed to several factors such as the presence of nutritional proteins and growth factors, physiologic osmolarity, pH buffering system and the low bacterial content due to pasteurization process (11, 12). To date, none of the milk proteins has been tested individually regarding their possible usage as a transport medium.

Casein phosphopeptides (CPP) are derived from casein, which account for 80% of the total protein in bovine milk (13). They can form soluble organophosphate salts and may function as carriers for different minerals, especially calcium. CPPs have been shown to help prevent demineralization and to aid in remineralization of tooth enamel, by competing with dental plaque forming bacteria for calcium (14). Recent studies have focused on cytomodulatory and antibacterial properties of CPP (13, 15).

The purpose of this study was to investigate the efficacy of a commercial CPP-amorphous calcium phosphate (CPP-ACP) preparation in preserving cultured L929 fibroblast viability as a potential new storage media for avulsed teeth.

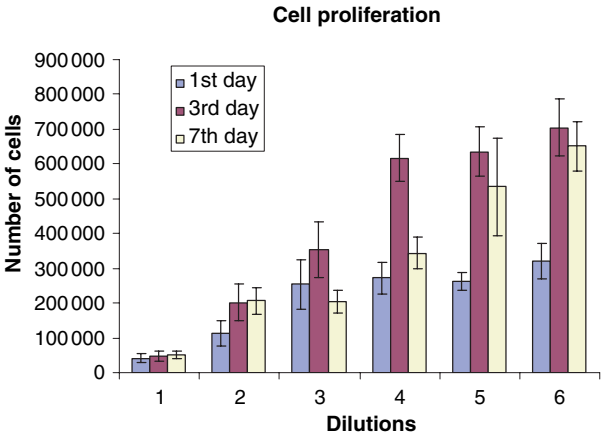

Fig. 1. Cell proliferation within time with respect to dilutions of the casein phosphopeptides-amorphous calcium phosphate (CPP-ACP) preparation. 1, 2, 3, 4, 5 and 6 represent 10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-12} dilutions of the CPP-ACP preparation and control respectively.

Materials and methods

Cell culture and assessment of cell viability

L929 fibroblastic cell line was plated in 24-well culture plates (Coastar Corp., Cambridge, MA, USA) at an initial density of 100.000 cells ml^{-1} in six replicas and incubated in Dulbecco's modified eagle medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA; supplemented with 10% Fetal Bovine Serum (FBS), 100 units ml^{-1} each of penicillin/streptomycin) in a humidified atmosphere of 95% air and 5% CO_2 for 12 h at 37°C. Following incubation, the cells were treated with 10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-12} dilutions (10^{-3} = 0.05 g

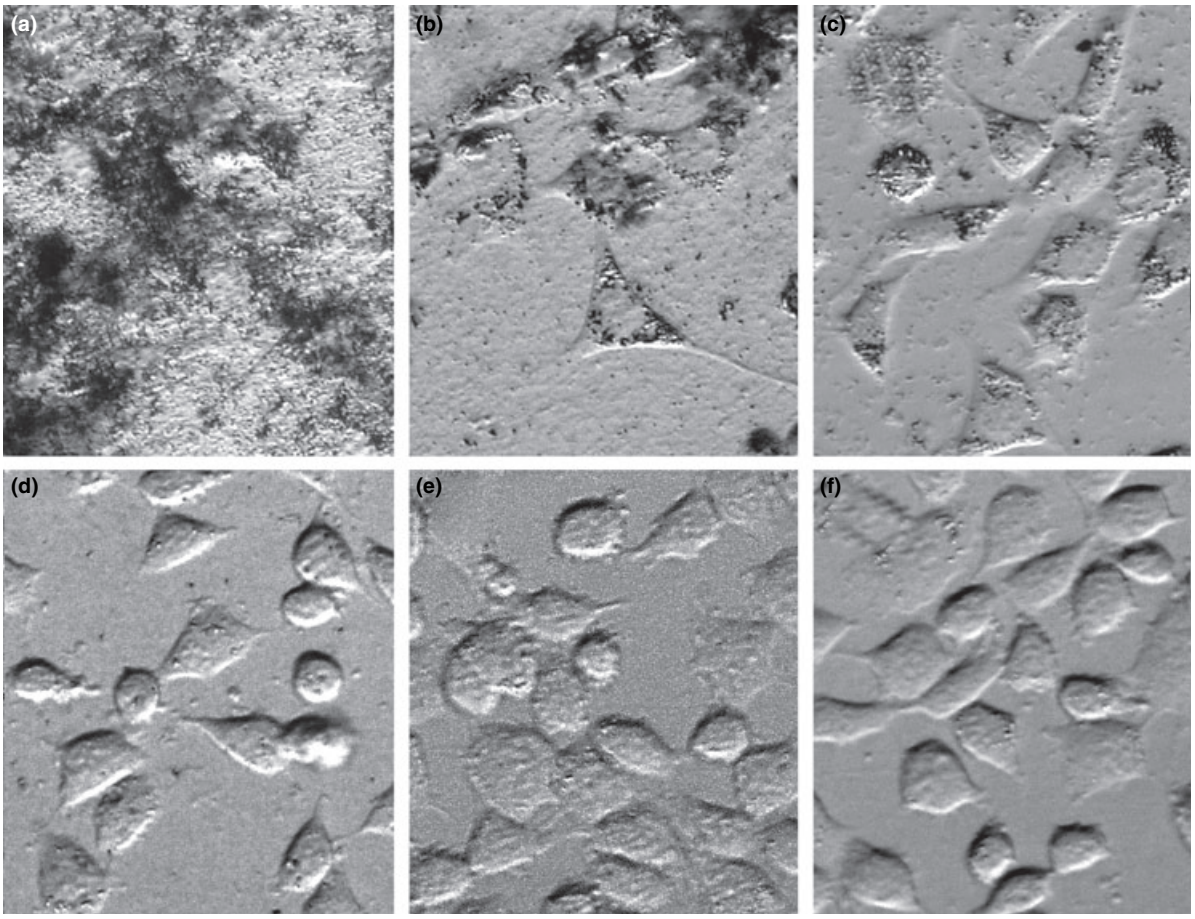

Fig. 2. Cell morphology at 24 h with respect to dilutions of the casein phosphopeptides-amorphous calcium phosphate (CPP-ACP) preparation. a, b, c, d, e and f represent 10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-12} dilutions of the CPP-ACP preparation and control respectively.

material per 100 ml medium) of a water-based CPP-ACP paste (Tooth Mousse, GC Corp., Tokyo, Japan) and the cultures were maintained for 7 days. Untreated cells served as controls. The L929 cells were counted at the 1st, 3rd and 7th days. At the end of each observation period, the culture medium was removed, and the cells were harvested using a mixture of 0.25% trypsin–EDTA. The effects of different concentrations of the CPP-ACP preparation on cell viability were assayed by cell counting. Cells were stained with trypan blue, and viable cells were examined as a percentage, using a hemocytometer under a light microscope (Olympus, Tokyo, Japan).

Assessment of apoptosis

Propidium iodide/acridine orange (PI/AO) staining was used to assess apoptosis of treated cells and of the positive control. At each evaluation period, the cell culture medium was removed and the cells on coverslips were washed briefly in sterile phosphate buffered saline (PBS). Approximately 25 μg ml^{-1} acridine orange (Sigma Chemical) and 25 μg ml^{-1} propidium iodide (Sigma Chemical) were mixed at a v:v ratio of 1:1 and added to the cells for 20 s. Thereafter, cells were washed in PBS for 10 s and mounted in PBS: glycerol (v:v, 1:1). The cells were visualized by fluorescence microscopy (Olympus AX70, Japan). Apoptotic cells were evaluated by counting red cells with fragmented nuclei. Acridine orange-stained cells were observed under a narrow band fluorescein isothiocyanate (FITC) filter (520–560 nm) in green colour, and propidium iodide-stained cells were observed under rhodamine filter (510–560 nm) as stained red. Cells were visualized and photographed under the fluorescence microscope at 40×.

Statistical analysis

For each concentration (dilution), statistical analysis of cell survival within time was performed using two-way analysis of variance (ANOVA). One way ANOVA and Tukey tests were applied to compare the effect of different concentrations on cell survival at each evaluation day. The comparison was made between control and 10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-12} dilutions of the CPP-ACP preparation, and between each dilutions of the material. All statistical tests were run at 5% level of significance.

Results

Cell viability

For each dilution tested, cell viability with respect to the evaluation periods is presented in Fig. 1. Except for the

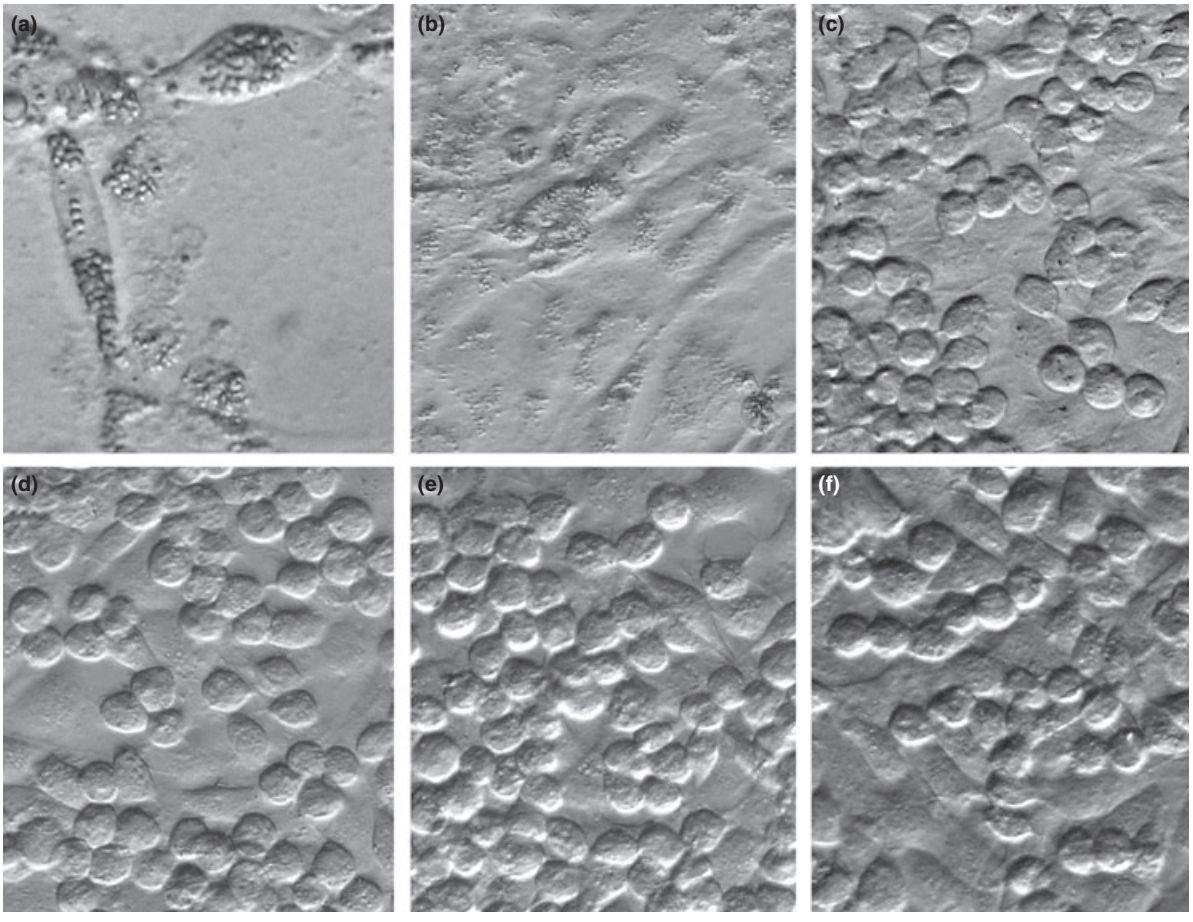

Fig. 3. Cell morphology at day 7 with respect to dilutions of the casein phosphopeptides-amorphous calcium phosphate (CPP-ACP) preparation. a, b, c, d, e and f represent 10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-12} dilutions of the CPP-ACP preparation and control respectively.

$10^{-3}$and 10^{-4} dilutions, all groups demonstrated an increase in cell numbers at days 1 and 3, followed by a decrease at day 7. Irrespective of the increase or decrease in cell viability, time-dependent changes for each dilution group were significantly different ($P < 0.05$, Fig. 1).

At day 1, the cell viability between $10^{-3}$and 10^{-4} dilutions of the CPP-ACP preparation was not significant ($P > 0.05$). Both groups, however, demonstrated significantly lower cell counts than the 10^{-6}, 10^{-8} and 10^{-12} dilutions, and the control ($P < 0.05$). Pairwise comparisons between the latter four groups did not show any significant difference ($P > 0.05$). At day 3, the 'toxic threshold' decreased to 10^{-6} ($P < 0.05$), with the higher dilutions (10^{-8} and 10^{-12}) and the control group demonstrating significantly greater number of cells ($P < 0.05$, Fig. 1). Pairwise comparisons between the latter three groups did not show any significant difference ($P > 0.05$). Despite the overall decrease in the number of vital cells at day 7, the highest dilution (10^{-12}) of the CPP-ACP preparation and the control groups demonstrated significantly greater number of cells, compared to the lower-dilution groups ($P < 0.05$) and the 'toxic threshold' decreased to 10^{-8} ($P < 0.05$).

Morphological assessment

Cell morphology

At day 1, a severe toxic response was observed morphologically in the $10^{-3}$and 10^{-4} dilution groups, due to the deposition of the CPP-ACP particles over the cells (Figs 2a and 1b). As a result of dilution, cells were able to survive in the 10^{-6} dilution group, and the CPP-ACP particles that were still observed in the medium did not interfere with cell growth and morphology (Fig. 2c). Cells in the 10^{-8} and 10^{-12} dilution groups demonstrated similar fibroblastic morphology as with the control group (Fig. 2d–f respectively). At day 7, the $10^{-3}$and 10^{-4} dilution groups did not demonstrate any difference with regard to cell number and morphology (Fig. 3a,b). Cells in the 10^{-6}, 10^{-8} and 10^{-12} dilution groups revealed similar morphological appearance with the control group (Fig. 3c–f respectively) although a tendency to detach from the surface was evident in all groups due to cell confluence.

Apoptosis

Starting from the first day of incubation, cells in the $10^{-3}$and 10^{-4} dilution groups demonstrated a rapid

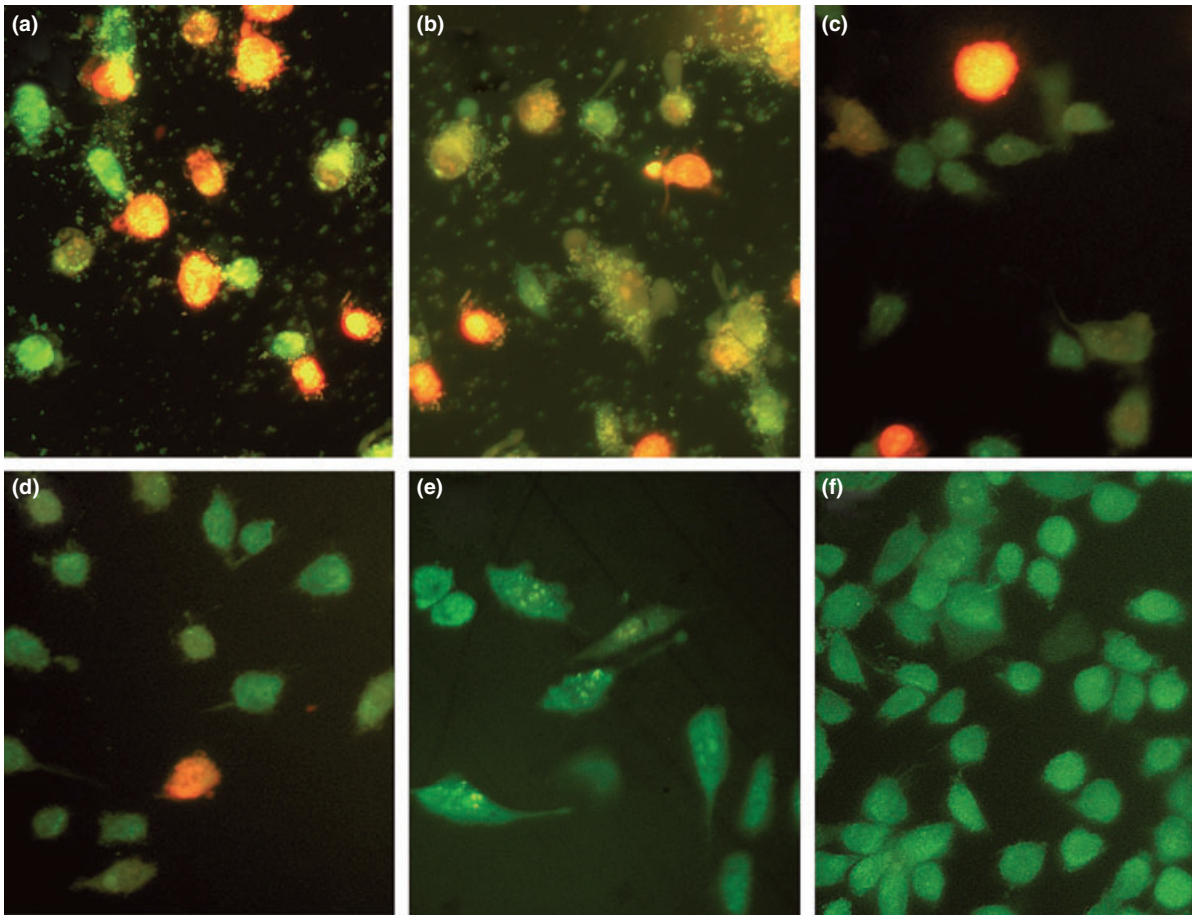

Fig. 4. Morphology of L929 cells with respect to apoptosis at 24 h. a, b, c, d, e and f represent 10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-12} dilutions of the casein phosphopeptides-amorphous calcium phosphate (CPP-ACP) preparation and control respectively.

apoptotic response, as evidenced by yellow, orange and red-staining, round cell morphology and membrane blebbing (Fig. 4a,b). A relatively few number of apoptotic cells were observed in the 10^{-6} and 10^{-8} dilution groups (Fig. 4c,d), while no sign of apoptosis was evident in the 10^{-12} dilution group and control (Fig. 4e,f respectively). Despite a relative increase in cell number at day 7, apoptosis was consistently observed in the $10^{-3}$and 10^{-4} dilution groups, showing typical staining and morphological patterns (Fig. 5a,b). As a result of cell ageing, apoptosis was also observed in the 10^{-6}, 10^{-8} and 10^{-12} dilution groups and control, but to a lesser extent (Fig. 5c–f respectively). Cells in the 10^{-12} dilution group (Fig. 5e) showed similar fibroblastic morphology as with those of control (Fig. 5f).

Discussion

In vitro cytotoxicity assays can be used as a first level risk assessment when working with new biomaterials (16). A variety of assays exist (17) and, in general, these assays produce approximately equivalent results when used to determine the tissue response of novel biomaterials (18). Most current tests determine the level of cell survival following exposure to a compound vs the same amount of unexposed control cells (17).

In the dental literature, various techniques have been utilized to quantitate the number of viable cells. Trypsinization procedure, followed by several staining techniques has been used for determining the viability of PDL cells in simulated avulsion injuries (12, 19, 20). In the present study, the trypan blue exclusion staining technique was chosen because it is fast, reliable, and distinctively differentiates non-viable cells from viable cells. Cell viability can also be evaluated by MTT (dimethylthiazoldiphenyltetrazol bromide; thiazolil blue) colorimetric assay (21). However, the status of the viable cells and their ability to proliferate cannot be accurately determined with dye exclusion or MTT assays. Thus, a cell apoptosis assay was used to determine the condition of the cells after their exposure to the different concentrations of the tested CPP-ACP preparation.

In the present study, the L929 fibroblast cells were chosen to demonstrate biocompatibility as they are a well, established, distinct line and genetically identical to each other. Thus, the experiment is reproducible and standardized. Primary gingival fibroblasts or osteoblasts that have been used in other experiments *in vitro* do not possess these properties and variable results are expected as the lineage is inconsistent. The behaviour of L929 cells has been compared with gingival fibroblasts; their physiology and adhesive properties have been shown to be similar (17).

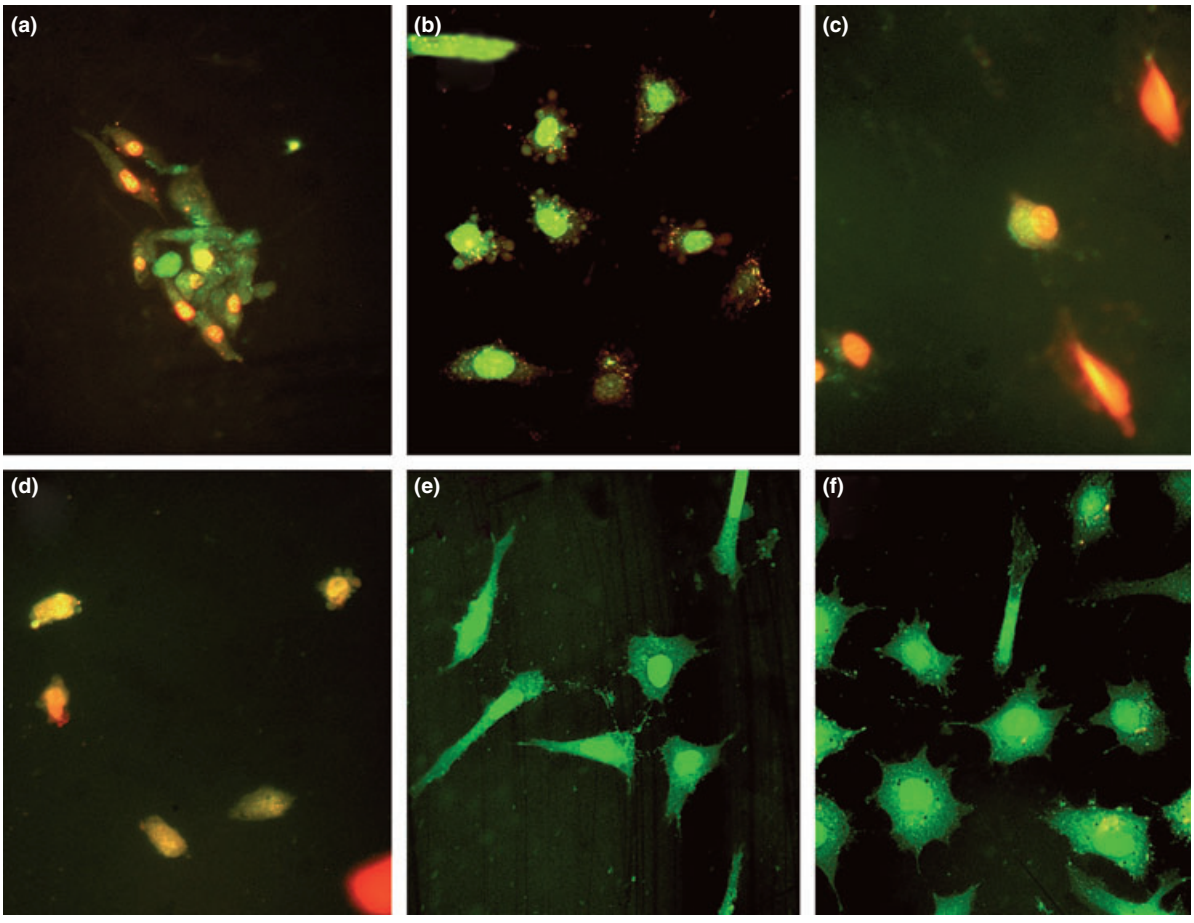

Fig. 5. Morphology of L929 cells with respect to apoptosis at day 7. a, b, c, d, e and f represent 10^{-3}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-12} dilutions of the casein phosphopeptides-amorphous calcium phosphate (CPP-ACP) preparation and control respectively.

The present study demonstrated that lower concentrations of CPP-ACP can be effective in maintaining cell viability *in vitro*. Indeed, stronger dilution of CPP-ACP was effective on both the viability and the morphology of the L929 cells, as the 10^{-8} and 10^{-12} dilution groups' demonstrated similar fibroblastic morphology and apoptotic response as with the control group.

On the other hand, fibroblasts in the 10^{-3} and 10^{-4} dilution groups demonstrated a severe toxic and apoptotic response starting from the first day of incubation and continued thereafter. One reason for this finding may be the CPP-ACP-dependent increase in the calcium (Ca^{2+}) concentration in the culture medium. While Ca^{2+} may trigger mechanisms required for cell proliferation, it can also stimulate cytosolic enzymes executing apoptosis (22). Moreover, due to complex interaction with other signalling pathways, a given ion channel may play a dual role in both cell proliferation and apoptosis (22, 23). It is also possible that early cell death and apoptosis in the 10^{-3} and 10^{-4} dilution groups could be due to the toxic effects of other ingredients/additives (i.e., glycerol, propylene glycol, phosphoric acid, etc.) (24) of the tested CPP-ACP preparation, which is not primarily manufactured as a tissue transport medium. It is, thus, logical to expect a decrease in the possible toxic/apoptotic effects of such ingredients as the dilution factor increased.

In the present study, the effect of the CPP-ACP was dose-dependent. Further research is needed to elucidate the potential beneficial effects of CPP-ACP treatment *in vivo*, and to quantify its optimal concentration for the preservation of the PDL cell viability of avulsed teeth.

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