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Research Article

Effect of Keratin Preparations on Cementoblast OCCM-30 and Fibroblast L929 Cells

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Abstract

Aim: To assess the *in vitro* effect of keratin preparations on the viability and proliferation of cementoblast OCCM-30 and fibroblast L929 cells and on the mineralization capability of the OCCM-30 cells.

Methodology: Cells of L929 and OCCM-30 were assessed for viability in growth media supplemented with 10, 1 and 0.1 mg/ml of keratin using a LIVE/DEAD assay and confocal laser scanning microscopy. Cell proliferation was tested with similar keratin concentrations using an alamarBlue® proliferation assay at 0, 24, 48 and 72 h. Alkaline phosphatase assay was performed for OCCM-30 cells with keratin concentrations of 1 mg/ml, 0.1 mg/ml at 3, 6, and 10 days and an alizarin red assay was performed at 14 days.

Results: The viability assay showed concentrations at 1 mg/ml or greater of keratin proved toxic to L929 cells. The proliferation assay for OCCM-30 cells showed keratin concentration at 10 mg/ml prevented its proliferation and was significantly lower than the other keratin groups ($P < 0.0001$). There was significantly higher OCCM-30 cell ALP activity with the control and 0.1 mg/ml groups compared with the 1 mg/ml group at 6 d and 12 days ($P < 0.0001$). The alizarin red assay was consistent with the ALP activity of the OCCM-30 cells.

Conclusion: Low concentration keratin preparations were shown to allow normal cell proliferation of L929 and OCCM-30 cells and normal mineralization of the cementoblasts.

Keywords: Bioscaffold; Cementoblasts; Fibroblasts; Keratin; Mineralization; Viability

Introduction

The unique composition of the tooth provides a structure that survives the daily routine of heavy abrasive forces, various exogenous chemical insults and varying temperature changes. However, teeth are susceptible to damage from trauma, bacteria and high acidic diets. Conventional treatment involves using restorative materials, root fillings or prosthesis to replace the missing tooth structure. The replacement is only a substitute and does not have the same architectural design or functional capabilities as the lost tissue [1]. Regenerative treatment may be a possible treatment option in the future. The basis for regenerative dentistry can be accomplished by using stem cells, tissue-engineering materials such as customized scaffold and growth factors, the body's own healing power, or a combination of these [2,3].

Several studies have shown that cementum has the capability to repair itself when damaged or lost [4-7]. Cellular intrinsic fiber cementum in particular has been shown to be rapidly deposited in thick layers, demonstrating its function as an adaptive and reparative tissue [8]. Various research projects are being undertaken using a regenerative approach, where the goal is to replace damaged or lost tissue with another exemplifying identical architecture designed to function as the original.

In context, a regenerative endodontic procedure would entail replacing damaged structures involving the crown, root or even the pulp-dentine complex [9] and it is considered as one of the most exciting new developments in dentistry. An appropriate scaffold that can accommodate cells responsible for regeneration is considered a vital step in order to achieve regenerative endodontic treatment. According to Galler and D'Souza [1], scaffolds provide a three-dimensional environment to stimulate growth and differentiation of cells. The scaffold material should be biocompatible and non-toxic for an application to be successful. In particular reference to endodontics, the scaffold must biodegrade as the cells are proliferating thus leaving behind no remnants of the scaffold itself over time. Injectable scaffold may seem promising due to the ability of delivering it directly into the root canal system. Keratin is one such protein that has shown biocompatibility in an osseous environment with osteoblastic cells as well as with neurons [10].

Keratins are structural proteins often found in vertebrate hair, wool, horn, feathers and other epithelial coverings. The potential of keratin has been explored a great deal based on its intrinsic ability to interact with different cells, and hence more techniques are being tested for its extraction and suitable reinforcement [11-14]. Keratin can be made into thin films, porous membranes, powder and gels. The application of keratin was used successfully to test nerve regeneration [15,16], hemostasis [17], wound dressing [18] and histamine release [19]. Many applications of keratin in relation to cell adhesion, proliferation and the preservation of cell viability [20-23] proved that keratin holds strong potential as a scaffold in biomedical applications

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and tissue engineering.

Collagen and keratin display several similarities including alpha helix polypeptide chains with a high amount of small amino acid residues [24]. A study by Huang et al. [25] showed that proliferation of pulp cells in a collagen gel as possible. Many scaffold materials use collagen as the medium of choice, and the possibility of keratin as a scaffold medium for dental applications was found to be effective in enhancing the growth and differentiation of odontoblast- like cells [26] and dental pulp healing [27]. There are a number of endodontic situations where regeneration of cementum and periodontal ligament is important to clinical outcome, such as apexification, root perforation repair and apicectomy. Since cementoblasts have properties similar to osteoblasts we investigated the effect of keratin in solution on the proliferation and mineralization of cementoblast OCCM-30 cells.

Materials and Methods

Cells

Cementoblast cells of murine origin (OCCM-30) were obtained from the School of Dentistry, University of Michigan, USA as a gift. The cementoblast cells were maintained in DMEM. Mouse connective tissue fibroblast cell line L929 was bought from the European Collection of Cell Cultures (ECACC; Salisbury, UK). The cell line is a subclone of the original L strain established by W.R Earle in 1940 from the normal subcutaneous areolar and adipose tissue of C3H/An mouse.

Keratin preparation

New Zealand Merino wool with a mean diameter of 21 µm was cleaned, dried and defatted by soxhlet extraction using a 1:1 v/v mixture of hexane and dichloromethane. Cleaned wool (10 g) was mixed with 7M urea (180ml), SDS (Sodium dodecyl sulphate, 6 g) and β-mercaptoethanol (15ml) in a round-bottom flask (300ml) and agitated at 50°C for 12 hours. The mixture formed was filtered through stainless steel mesh and the filtrate dialysed using 10 kDa cut off membrane (Spectra/por, Spectrum labs, Shiga, Japan) against degassed water (3.5L) containing 0.08% β-mercaptoethanol for two to three times to create a colourless and clear solution of reduced solubilised keratin.

The resulting solution was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE mini gel, Invitrogen, Novex NuPAGE Bis-Tris gels) for quantification (molecular weight) of proteins. The Pierce™ BCA (Thermo Scientific, Illinois, USA) protein assay kit was used to measure the protein content of the obtained extract. The yield was ~ 47.26 wt % from the starting wool. A SDS-1D PAGE of the solution obtained two major bands. The bands were between 40-60 kDa indicative of the intermediate filaments (IFPs) and minor bands of 15-20 kDa suggestive of intermediate filament associated proteins (IFAPs) (Figure 1).

Protein powder from the solubilised protein extracts was obtained by lyophilisation (Labconco Freez-one freeze-dryer, Labconco, Missouri, USA). The characterized powder was sterilized by gamma-irradiation at a dose of 800 krad prior to cell culture.

Media preparation

Dulbecco's modified Eagle's medium (DMEM): DMEM was used for the OCCM-30 cell line. As per manufacturer's instructions, the media powder (Gibco™/ Life Technologies, Auckland, NZ) was placed into 950 ml water at room temperature with gentle stirring. Sodium bicarbonate was added to the media to 3.7 g/L. The pH was adjusted to 7.1 with the addition of 1N NaOH or 1N HCL depending on the pH level. The resulting solution was sterilised using positive pressure membrane filtration.

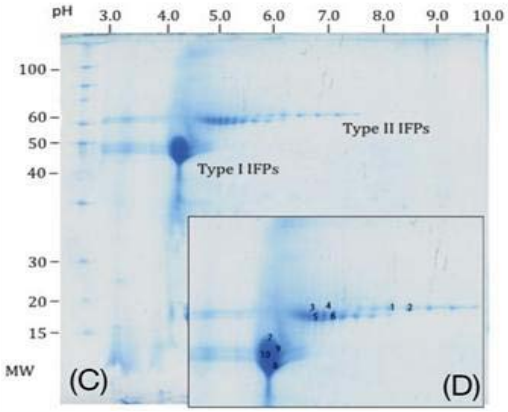

Figure 1: SDS-PAGE of freeze dried samples (n=2) shows consistent protein banding patterns (Type I 45-50 kDa, Type II 54–56 kDa; pIs 4.5-7.5 (D). Gel prepared for MALDI-MS/MS mass spectrometry which identified protein homologs consisting of 6 Type I (K33, 33a, 33b, 8c-1, 31, 34) and 6 Type II (K81, 83, 85, 86, 7c, 5) keratin intermediate filament proteins.

Minimum Essential Medium (MEM): MEM was used for L929 cells. The same procedure was followed as with the DMEM, but sodium bicarbonate was added to the media to 2.2 g/L.

Mineralization Media (MM): MM was made up of DMEM containing 5% FBS, 1% antibiotic-antimycotic, ascorbic acid (50 µg/ml) and β-glycerolphosphate (10 mmol/L).

Keratin media preparation

Keratin solutions for the experiments were created by diluting the keratin powder in DMEM or MEM. The highest concentration tested was 10 mg/ml keratin as pilot studies performed showed that greater than 10 mg/ml keratin resulted in gelation formation over time. The keratin powder (100 mg) and solution (10 ml) was mixed thoroughly with help of a vortex machine. The solution was kept in an incubator at 37°C, 5 % CO_2 then filtered using a syringe filter (0.22 µm) under sterile conditions. For the proliferation and viability experiments, serial dilution was performed from the 10 mg/ml solutions.

Analysis of the bioactivity of cells on keratin solution

Cell viability assay: The OCCM-30 and L929 cells were grown at 37°C in a 5% CO_2 atmosphere in DMEM or MEM supplemented with 10% Fetal Bovine Serum (FBS) (Gibco™/Life Technologies), 1% antibiotic-antimycotic (Gibco™/Life Technologies, Auckland, NZ) containing 10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, and 25 µg/ml of Fungizone® and likewise maintained on tissue culture polystyrene (Corning®/ Life Sciences, New York, USA). Cells were harvested from a sub-confluent culture by using 0.25% trypsin and counted with an automatic cell counter and seeded onto a 48-well plates at a density of 5 x 103 per well (500 µl/well). For viability experiments, cells were allowed to adhere for 24 h in

DMEM or MEM, after which the media was changed to DMEM or MEM with keratin (0.1, 1.0 or 2 mg/ml) and 10% FBS. Culture media supplemented without keratin served as the control group.

The Live/Dead assay (Molecular Probes™/ Life Technologies, California, USA) was used to determine the cytotoxicity after 24 h. A stock solution of 4 µM ethidium homodimer-1 (EthD-1) and 2 Calcein AM were made up from the 2 mM EthD-1 and 4 mM Calcein AM solution in a D-PBS buffer. The well plates were washed with 500 µl of D-PBS/well to remove or dilute serum esterase activity generally present in the culture medium. After sufficient rinsing with D-PBS, 100 µl/well of the Calcein AM and EthD-1 working solution was added to cover the bottom of the well containing the L929 or OCCM-30 cells. The solution was left in the incubator (37°C, 5% CO2) for 15 mins. Following which, the fluorescence (ex/em ~495 nm/~515 nm) for Calcein AM and (ex/em ~495 nm/ ~635 nm) for EthD-1 was imaged with an inverted confocal microscope (Zeiss LSM 510, Carl Zeiss Microscopy, Germany). All experiments were replicated three times with similar results.

The images were captured using Zeiss LSM 510 control software, version 3.2, running with Windows XP. The software ZEN lite 2012 (Carl Zeiss, Jena, Germany) was used for the confocal microscopy images processing and analysis. Several images were taken for each group at random locations on the well plate and one image deemed most representative of the viability activity of the cells was used. The image size scanned was 1.84 mm x 1.84 mm at a pixel time of 3.20 µs.

Cell proliferation assay

Following the same protocol as the viability experiment, the OCCM-30 cells and the L929 cells were seeded at a density of 6 x 103 per well (200 µl/well) in a 96-well plate and were tested with concentrations of 0.1, 1.0 and 10 mg/ml keratin. The alamarBlue assay (Biosource™/ Life Technologies) was then added to the wells at 10% volume of the original media (20 µl/well) to measure cell proliferation at 0, 24, 48 and 72 h. The alamarBlue assay was allowed to be metabolised by the cells in the incubator for 4 h. Following which, the fluorescence (excitation 540 nm and emission 610 nm) was measured using a Synergy™ 5 multi-detection micro plate reader (BioTek Instrument Inc., Northern Vermont, USA). All experiments were replicated three times.

Alkaline Phosphatase (ALP) assay

Quantitative analysis of ALP activity was detected with a SensoLyte® pNPP Alkaline Phosphatase Assay kit (AnaSpec/EGT Group, California, USA) following the manufacturer's protocol. The OCCM-30 cells were plated at 0.5 ml/well with a seeding density of 1000 cells/ml in DMEM containing 10% FBS on 48-well plates (6 well plates per group being tested). After 24 h, the culture media in the well plates was switched to DMEM containing 5% FBS + MM (ascorbic acid 50 µg/ml and β-glycerolphosphate 10mmol/L) plus the keratin dilutions 0.10 mg/ml and 1 mg/ml). The DMEM mineralization media without any keratin was used as the control. The media was changed every three days, and the ALP was determined after 3, 6 and 12 d using SensoLyte® pNPP.

Briefly, the cells were washed with 1x assay buffer from the assay kit. The assay detergent was used to break up the cells on the well plates. The cells and assay detergent were scraped off from the well plates into microcentrifuge tubes. The cell suspensions were incubated at 4°C for 10 mins under agitation and centrifuged at 2500 g for 10 mins at 4°C. The supernatant was collected for the ALP assay and transferred to a 96 well plate (50 µl/well). To determine the ALP level, 50 µl of SensoLyte® pNPP substrate solution was added to each well. After incubation of the well plates for 45 mins, the absorbance at 405 nm was measured using a Synergy™ 5 multi-detection micro plate reader (BioTek Instrument, Inc). The experiments were replicated twice.

To determine the level of ALP in the sample, an alkaline phosphatase standard was made up. A known concentration of ALP at 10 µg/ml was diluted to 0.2 µg/ml. A two-fold serial dilution was done to get a range of concentration from 100 ng/ml to 0.15 ng/ml. To determine the ALP level absorbance for the standards, 50 µl of SensoLyte® pNPP substrate solution was added to wells containing 50 µl of each of the standards. Prior to absorbance reading at 405 nm, the well plates were left to incubate for 45 mins and the absorbance was measured at 405 nm.

Alizarin red assay and quantification

Mineralization activity was assessed by growing the confluent cells in DMEM supplemented with 5% FBS and MM for 14 and 21 days, the cells were fixed with ice-cold 70% ethanol and stained with AR-S 40 mM (Sigma-Aldrich, Cat No. A5533-25G). Briefly, 3.42g of alizarin red s + 100 ml of dH2O (100 mM stock solution), (40 mM/100mM)*100 ml = 40 ml (100mM stock solution) + 60 ml (dH_2O) under shaking produced 40 mM AR-S. The pH was adjusted to 4.2 with ammonium hydroxide solution. The wells for 21 d were incubated without replenishment of media from day 18 to allow the cells to undergo necrosis. This allowed the alizarin red to stain exclusively the mineralised mass attached to the well plate rather than the cells and extracellular calcium present.

The well plates were rinsed with mH_2O five times and a final rinse with PBS for 15 mins under shaking to remove the unspecific bound alizarin red. The stained cells were photographed prior to quantification of the stained alizarin red. For quantification, each well plates were incubated with 1 ml of 10% (w/v) cetylpyridinium chloride (CPC 358 g mol-1, Sigma-Aldrich, Cat No. C0732-100G) in 10 mM sodium phosphate, pH 7.0, for 20 mins at room temperature and gentle shaking. The cells stained with AR-S were de-stained with CPC and the extracted stain was transferred to a 96-well plate (200 µl/well). The absorbance at 562 nm was measured using a Synergy™ 5 multi-detection micro plate reader.

An alizarin red standard curve was generated using the 40 mM alizarin red solution. A 1:20 dilution buffer was made up by diluting 50 µl alizarin red with 950 µl of the 10% CPC in 10 mM sodium phosphate buffer to give a 2 mM working solution of alizarin red. A high range set was made up by diluting the 2 mM alizarin red working solution in a 2-fold serial dilutions (2 mM to 0.06 mM). A low range set was made up by diluting the 2 mM stock alizarin 1:66 to give a solution of 30 µM working stock. The 30 µM solution was further diluted in a two-fold serial dilution (30 µM to 0.9 µM).

Statistical analysis

For proliferation and ALP experiments, two-way ANOVA analysis and Tukey's multiple comparisons test were used, for alizarin

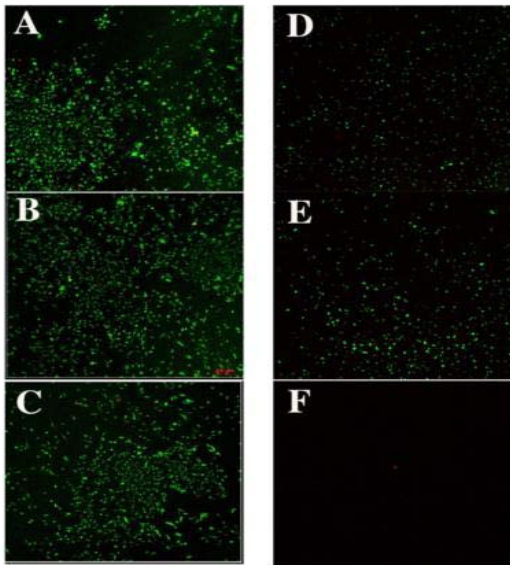

Figure 2: Cell viability of OCCM-30 cells (Figure 1A, B, C) and L929 cells (Figure 1 D, E, F) with keratin preparations: control (Figure 1A, D), 0.1 mg/ml (Figure 1B, E) or 1mg/ml keratin (Figure 1 C, F). Scale Bar (B) 200µm.

red experiment, one-way ANOVA were performed (GraphPad Prism 6.0). A confidence level of $p < 0.01$ was used for statistical analysis.

Results

Cell viability assay

Cell viability of OCCM-30 cells subjected to 0.1 mg/ml or 1mg/ml keratin preparation and L929 cells with 0.1 mg/ml keratin for 24 h was similar to the control ($p > 0.01$) and showed an even distribution of viable cells across the well plate (Figure 2). On the other hand, L929 cells exposed to 1 mg/ml of keratin showed no signs of viable cells ($p < 0.01$) and very few adherent cells, suggesting that fibroblast cells were unable to bind to the surface at this keratin concentration. Exposure of OCCM-30 cells to 2 mg/ml of keratin showed very few viable cells ($p < 0.01$) adhering to the surface, suggesting inhibition of cementoblast cell adhesion at this keratin concentration. At keratin concentrations above 2 mg/ml no cementoblast cells were seen to adhere to the well surface.

Proliferation assay

The different concentrations of keratin solutions produced different fluorescence proliferation values for the OCCM-30 cells and L929 cells (Table 1, Figure 3). The mean fluorescence values increased from 0 h to 72 h for OCCM-30 cells up to 1 mg/ml keratin concentrations and 0.1 mg/ml for L929 cells ($p < 0.01$). Above these concentrations there was no cell proliferation reflecting the viability of cells as above.

Mineralization activity of OCCM-30 cells

At keratin concentration of 1 mg/ml, OCCM-30 cells failed to show any signs of mineralization activity at the different time points (3, 6 and 12 days). At 3 days OCCM-30 cells with 0.1 mg/ml of keratin did not show any ALP. However, ALP activity was evident ($P < 0.001$) at 6 and 12 days for the 0.1 mg/ml group and showed similar levels to the control group ($P > 0.01$) on either of the days. This corresponded to the similar number of cells/proliferation between these groups ($p > 0.01$) at the time points (Table 1, Figure 3). The results showed a significant ($P < 0.01$) increase in ALP activity for the 0.1 mg/ml group from 10.3 ng/ml at 6 days (control group 7.1 ng/ml) to 168.73 ng/ml at 12 days (control group 170.37 ng/ml) (Figure 4), with values conforming to a standard curve.

The level of mineralization was verified by AR-S staining. There was no uptake of the alizarin staining at 14 d for OCCM-30 cells subjected to 1 mg/ml of keratin solution whereas staining was clearly observed in the control and 0.1 mg/ml keratin solution groups (Figure 5). The wells for 21 d were incubated without replenishment of media at 18 days to allow the cells to undergo necrosis. This allowed

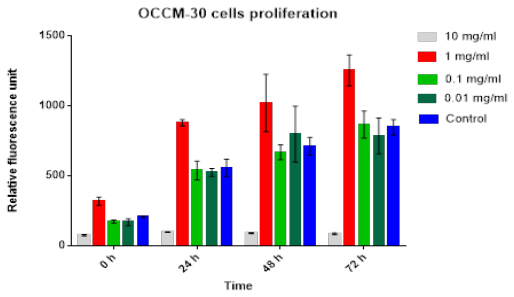

Figure 3: Graph of mean fluorescent units at various times of OCCM-30 cells after exposure to keratin solution.

Table 1: Mean fluorescent units for OCCM-30 and L929 cells at various keratin concentrations and times. Two-way ANOVA analysis and Tukey's multiple comparisons tests similar superscript letters indicate significant difference at $P < 0.01$ level.

OCCM-30 cells				
Keratin	0 h	24 h	48 h	72 h
10 mg/ml	76[a]	96	90	85
1 mg/ml	319[a]	878[b]	1020[c]	1253[c]
0.1 mg/ml	173	538	668	866
0.01 mg/ml	168	524	798	786
control	207	556[b]	711	847
L929 cells				
10 mg/ml	94	91[e]	118	85
1 mg/ml	216	93[f]	118	123
0.1 mg/ml	254	766	1412[g]	1447[h]
0.01 mg/ml	223	696	946	1273[i]
control	167	495[def]	977[g]	1123[hi]

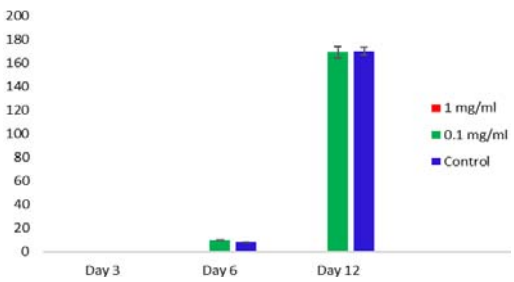

Figure 4: ALP activity (ng/ml) of OCCM-30 cells exposed to keratin solutions for different times.

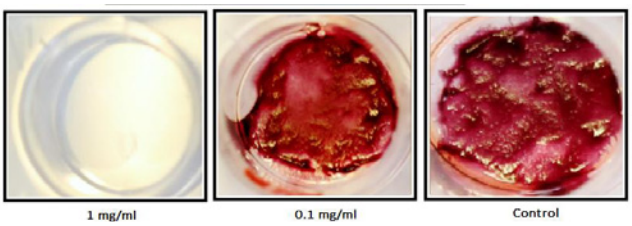

Figure 5: Alizarin stained OCCM-30 cells at day 14 exposed to keratin solutions.

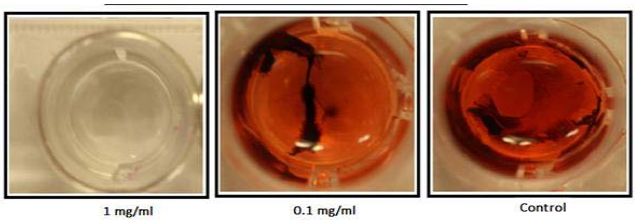

Figure 6: Alizarin stained well plates at day 21, after incubation without replenishment of media at day 18 to allow the OCCM-30 cells to undergo necrosis, demonstrating mineralised mass attached to well.

the alizarin red to stain exclusively the mineralized mass attached to the well plate rather than the cells and extracellular calcium present (Figure 6).

Quantitative de-staining of the alizarin staining using 10% (w/v) CPC solution in 10 mM sodium phosphate showed consistent results similar to the ALP assay. There was minimal (< 10 μM) alizarin binding to calcium ions at 1 mg/ml keratin. The mean reading for OCCM-30 cells subjected to 0.1 mg/ml of keratin solution was 72.8 μM (range 66.78-75.67 μM) and 78.2 μM (range 67.89-80.1 μM) for the control group and 4 μM (range 0-6.7 μM). There was significantly ($P < 0.0001$) less uptake of stain in the 1 mg/ml group compared to 0.1 mg/ml and control groups. There was no significant difference ($P > 0.01$) between the 0.1 mg/ml and the control groups.

Discussion

This study was conducted to evaluate the effect of keratin preparations on the viability and proliferation of cementoblast OCCM-30 and fibroblast L929 cells and the mineralization capability of OCCM-30 cells. It revealed that various concentrations of keratin solution affected the cells proliferation and viability. A keratin concentration of 1 to 10 mg/ml showed no signs of L929 cell proliferation or viability compared to concentrations of 0.1 mg/ml of keratin and below. This is in contrast to a study conducted by [28] on L929 cells where a 10 mg/ml keratin hydrogel showed promising results. While Wang et al. [28] showed a greater quantity of live cells staining the control group compared to keratin. In contrast, the current study showed growth of L929 cells similar to the control group for cells subjected to keratin concentrations of 0.1 mg/ml or lower. These differences may be partly explained in that the Wang et al. [28] experiment was conducted using keratin as a gel rather than a solution and compared results to a collagen hydrogel, which showed cell proliferation was more abundant on the collagen hydrogel.

The future application on the use of keratin as a biomaterial is aimed at being a resorbable material thus lower rather than higher concentrations should be tested. In the present study, concentrations greater than 1 mg/ml of keratin solution proved to be toxic to OCCM-30 cells after 24 hours. Since the isolation of the OCCM-30 cell lines several other biomaterials have been tested against cementoblast cells [29,30]. The results from *in vitro* tests against cell lines should be taken with care as minor changes in the material tested against the same cell line may show variation. Use of various concentrations of MTA has been researched as a biomaterial for endodontic applications with OCCM-30 cells *in vitro* [31]. Moreover, Oviir et al. [31,32] tested the effect of grey and white MTA on the proliferation of OCCM-30 cells and showed white MTA to be more effective. A study by Sierpinski et al. [15] found proliferation of RT4-D6P2T Schwann cells were enhanced by the use of keratin. Their results showed inhibition of RT4-D6P2T cells proliferation at keratin concentration of 10 mg/ml. In contrast, lower concentrations showed significant increases in proliferation similar to the control group which is consistent with the current study.

The current study showed there is a difference between the cell lines in response to the various concentrations of keratin. The L929 cell showed more sensitivity to the keratin with no sign of proliferation at 1 mg/ml of keratin solution compared to OCCM-30 cell which responded favorably to keratin at 1 mg/ml. The difference between the L929 and OCCM-30 results and the other studies might be explained by the use of different cell types and by the dissimilar experimental designs. Furthermore, the absolute influence of keratin and the concentrations used in the current study cannot be determined from the current results of *in vitro* cytotoxicity tests as variables such as the method used to make the keratin could influence the cell proliferation.

The role of ALP as a marker for osteogenic activity has been consistently established [33]. For the current study, only concentrations of keratin 1 mg/ml and below were subjected to OCCM-30 cells, as proliferation and viability experiments showed toxicity above 1 mg/ml keratin concentration. Levels of ALP detected at 3 days were similar for 1 mg/ml and 0.1 mg/ml keratin groups and the control group, indicating that initial cell growth tolerated the level of keratin. However, by 6 and 12 days, the 1 mg/ml keratin concentration did not allow OCCM-30 cells to express ALP which was at odds with the proliferation and viability experiments. The OCCM-30 cells may have undergone cell death prior to the 6 d reading of the ALP activity, due to contact inhibition and confluence-induced apoptosis. Changing the media more regularly and culturing the cells in larger well plates may have shown different results. Thus the ALP activity needs to be further assessed for OCCM-30 cells subjected to 1 mg/ml of keratin concentration before concluding whether it was toxic to OCCM-30 cells. The current ALP study was done with a cell seeding density of 500 cells per well. Hence, further experiments should be conducted with a lower cell count to rule out cell death of OCCM-30 cells exposed to 1 mg/ml keratin. As an early marker of cementoblast differentiation, ALP usually plays a role in transferring the phosphate groups from the cells to the matrix. The ALP activity at 6 days of OCCM-30 cells subjected to 0.1 mg/ml keratin showed detectable levels of alkaline phosphatase at 10 ng/ml, while at 12 days, it had more than a 10-fold increase compared with 6 days. Therefore 0.1 mg/ml keratin did not appear to hinder the differentiation of OCCM-30 cells to undergo mineralization. The study cannot be used

to show keratin-inducing alkaline phosphatase activity in a dose and time-dependent manner. The study can conclude that ALP activity of OCCM-30 cells is not affected in a detrimental manner at a keratin concentration of 0.1 mg/ml.

The ALP activity of OCCM-30 cells showed consistent results with the alizarin staining. However, there are limitations to the destaining of alizarin with CPC, for example a study by Gregory et al. [34] showed the CPC method to be less sensitive with reduced signal to noise ratio, especially for weakly stained monolayers of alizarin red. A study by Chun et al. [35] assessing bisphosphonate behavior of OCCM-30 cells from transgenic mice showed less ALP activity associated with cementoblast compared to osteoblast cells. Hence, future studies should assess cell specific factors unique to a cell line.

Certain limitations exist when interpreting results gained from *in vitro* studies to identify and extrapolate to cell specific effects on the function of basal cells. Mammalian cells connect not only to each other, but also to extracellular matrix. Due to this complex mechanical and biochemical interplay, research involving cells in a flat layer can miss subtle biological behavior otherwise seen in a three-dimensional tissue [36]. The assessment of the significance of the *in vitro* tests to an *in vivo* situation poses another major obstacle. Factors that influence *in vivo* tests, such as rate of absorption, biotransformation, distribution and excretion, cannot be replicated in an *in vitro* test. The challenge with confocal microscopy examination of living cells is obtaining an image without upsetting the cells. Variables such as temperature, CO_2, or pH cannot be maintained during the confocal imaging procedure to what the cells were accustomed to during their incubation. The imaging system must obtain the data within the time period before dramatic changes occur to the cells being tested including cell death.

The alamarBlue assay was used for the cell proliferation study and the culture medium had fetal bovine serum incorporated which has been shown to cause quenching of the assay. Furthermore, microbial contamination can reduce the alamarBlue dye and create false positive signals. Hence the assay has to be carried out under aseptic conditions and the medium may require antibiotics to eliminate the microbial contamination [37] and shorter exposures [38]. Alkaline phosphatase is among the first functional genes indicted in the calcification process. However, the use of assays to measure ALP activity has limitations. Even with minor cell density, the ALP activity quickly exceeded the reference range of the spectrophotometer in 12 days for OCCM-30 cells subjected to 0.1 mg/ml of keratin concentration and the control group.

Future studies need to determine the cut-off level for toxicity of the keratin concentrations. A different assay that measures the cells that are not bonded to the well but are still viable may show different results than the viability assay used in the current study and can be explored further.

Conclusion

Keratin has the potential to be used as a tissue regeneration scaffold material. Within the limits of this *in vitro* study, low concentrations of keratin in solution did not alter the cell behavior of fibroblast L929 cells and cementoblast OCCM-30 cells.

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