Comparing the Adhesion Capability of Periodontal Ligament Fibroblast Cells to Nano-hydroxyapatite Silicate-Based Cement and Silicate-Based Cement Alone

Hooman Khorshidi¹, Shahab Honar¹, Saeed Raoofi¹, Negar Azarpira²

¹Department of Periodontics, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran
²Department of pathology, school of medicine, Shiraz University of Medical Sciences, Shiraz, Iran

Received 11 February 2021 and Accepted 12 March 2021

Abstract
Introduction: Silicate-based cement alone and Hydroxyapatite as bone filling materials lead to successful results in implant dentistry and regenerative medicine. The purpose of this study was to compare the adhesion capability of periodontal ligament fibroblast cells (PDLFC) to the Nanohydroxyapatite silicate-based cement and silicate-based cement alone in vitro.

Methods: Primary cell cultures of PDLFCs were obtained from clinically healthy third molars teeth. These third molars were either extracted for orthodontic reasons or extracted due to the impaction of teeth. Cells subcultured at a density of 10000 cells/well in 24-well plates. Methyl-tetrazolium bromide (MTT) assay was performed to evaluate the survival and proliferation of fibroblasts on 24h, 72h, and 1 week after the cell culture. Scanning Electron Microscopy (SEM) analysis was used to examine the morphology of PDLFCs on the two scaffolds.

Results: Cells were found growing in close proximity to both minerals but in terms of fibroblast cell attachment. Adding Nanohydroxyapatite did not improve cellular proliferation and silicate-based cement alone showed superior cellular proliferation in 72 hours. After 24h and 1 week both minerals showed the same response.

Conclusion: Although both Nanohydroxyapatite silicate-based cement and silicate-based cement alone are biocompatible, but nanohydroxyapatite silicate-based cement did not show improved biological activities when compared with silicate-based cement.

Keywords: Apc cement; Bioceramics; Cell proliferation; Nanobiomaterials; Regeneration
Materials and Methods

Periodontal ligament fibroblast cells culture

Ten third molar teeth (extracted from healthy subjects due to non-pathological problems) were collected and placed in a basic medium [α-MEM (Minimum Essential Medium α) supplemented with 10% FBS (fetal bovine serum)]. The samples were transferred to the laboratory at 4 °C. The PDL tissue separation was performed under sterile conditions, within a biohazard laminar flow hood for each tooth. Tooth surfaces were first cleaned with 70% ethanol. A scalpel blade was used to remove PDL from the root surface. Tissue was cut into small pieces (smaller than 0.5 mm) and cultured in cell culture plates in DMEM containing 10% fetal bovine serum and 0.5% antibiotics (diluted from a stock solution containing 5000 U/ml penicillin and 5000 U/ml streptomycin) at 37° C in an atmosphere of 100% humidity and 5% CO2. The cells that grew were detached from the culture flask using 0.05% (w/v) trypsin and 0.05 mM (w/v) EDTA and transferred to 24-well plates where they were subcultured at a density of 10000 cells/well.

2.2. Cell suspension and Counting

The 0.5 ml of cell suspension was taken using a sterile pipette and placed in an Eppendorf tube. 100 µL of cells were transferred to another Eppendorf tube and 400 µL of Trypan Blue 0.4% was added and slowly mixed.

Pipettes were used to obtain 100 µL of trypan blue treated cell suspension and applied to a hemocytometer. The microscope was used to focus on homocytometer grid lines with a 10-fold objective. Live cells (Live cells do not stain with Trypan Blue) were counted in a set of 16 squares.

For determining the number of cells/ml and the total number of cells the following formula was used:

\[
\text{cell/ml} = \frac{\# \text{ of cells counted}}{\# \text{ squares counted}} \times 10000 \times \frac{1}{\text{dilution factor}}
\]

Total # cells = cells/ml × vol. of original cell suspension

To calculate the percentage of viable cells the following formula was used:

\[
\% \text{viability} = \frac{\# \text{viable cells counted}}{\text{Total # cells counted}} \times 100
\]

Experimental design

In this study we had 3 groups: Group 1 Silicate-Based Cement alone (APC): 90% Portland cement type 1(Fars Cement Co, Iran) with 10% Calcium Chloride (Kimia Material Co, Iran) additive - using a volumetric spoon. Group 2 Nanohydroxyapatite silicate-based cement (APC +Nano-HA): Based on previous experience (4), the ratio of the two materials was set to 25% APC in combination with 75% Nanohydroxyapatite (PardisPajouheshFananvaran Yazd, Iran) - using the volumetric spoon. Group 3 Control: PDLFCs seeding on a culture plate (as control). Each test group materials mixed with distilled water for setting reaction. APC was mixed with water at a 2: 1 ratio; APC +Nano-HA was mixed with water at a 3: 1 ratio. Scanning electron microscopy with a TESCAN scanning electron microscope (VEGA 3 – TESCAN, Czech Republic) at ×5k magnification was used to evaluate the surface morphology. Zeiss EM10C transmission electron microscope (TEM) at 80 kV (Zeiss, Germany) was performed to obtain the morphology and size of the nanoparticles.

The mixed test materials placed into 48-well plates (8-well plates for each material for 3 timing periods of 24h, 72h and 1 week). Each experiment was done triplicate.

MTT assay was used to evaluate the survival and proliferation of fibroblasts on days 24, 72 and 1 week after cell culturing. A fresh culture medium with 10% MTT was added to each well, and the plates at 37° C incubated for 4 hours. The culture medium of each well was extracted and replaced with dimethyl sulfoxide solvent. Then, 100 µl of each purple solution was transferred to a 96-well Elisa reader plate. The absorbance of the dye was measured by Elisa reader (POLARstar Omega, Germany) at 570 and 630 nm. Optical density (OD, absorbance) of wells was used to calculate the percentage of survival of cells in each experimental group relative to the control group.

\[
\text{Cell Survival} = \frac{\text{OD Test}}{\text{OD control}} \times 100
\]

The morphology of PDLFC in two scaffolds was examined by scanning electron microscopy (S-750, Hitachi, and Tokyo, Japan), then PDLFCs were seeded in each scaffold in 24 well plastic culture plates. Forty-eight hours after initial seeding, the culture medium was discarded and cells were washed slowly with a solution containing phosphate three times. Cells were fixed on scaffolds with 2.5% glutaraldehyde for 12 hours. The fixative removed and the scaffolds were carefully washed with phosphate-buffered solution.

Then the scaffolds were exposed for 15 minutes to sequential dehydration with a series of ethanol (50%, 70%, 80%, 90% and 95%). The isoamyl acetate replaced
and the scaffolds were allowed to dry at a typical critical point, then coated with gold using an ion-sputtering coater (IB-5, Eiko, Japan) and subjected to a scanning electron microscope for evaluation of cell attachment and morphology.

Data analysis

In the MTT analysis, mean values and standard deviation were calculated for each group. Differences for each material between the three-time periods tested according to the analysis of variance (ANOVA) and one-way analysis of variance were also performed for the material with significant difference by ANOVA. Differences between the two substances for each time period were assessed using Student-t test. The significance level was considered to be 0.05.

Figure 1. SEM image of APC+nano-HA substrate. Note the needle-like Hydroxyapatite crystals

Figure 2. Transmission Electron Microscopy (TEM) of Hydroxyapatite Nanoparticles

Result

Table I shows MTT data analysis and statistics of two different substrate (APC&APC+nano-HA) for 24, 72 h, and 1 week. In terms of cell population, data analysis of MTT assay reflects no statistically significant difference between APC & APC+nano-HA groups at 24 h and 1 week (P = 0.511 & 0.162 respectively). Although there is a significant difference between APC & APC+nano-HA groups at 72 h for the benefit of the APC group (p=0.016).

Fig. 3 shows the cell number on the APC substrates determined by the MTT assay 24, 72 h, and 1 w after seeding. The cell population after 24 h reflects initial adhesion on the APC substrates, and the great increase in the population after 72 h indicates subsequent proliferation of the cells while there is a decline in cell population after 1 week to the level of the initial adhesion at 24 h. MTT data analysis shows statistically significant differences between three times. Table I and II. (P = 0.001)

On the other hand, the cell population of APC+nano-HA substrates after 24, 72 h and 1 week of seeding, reflects initial adhesion on the substrates with no statistically significant difference between three times. Table I. (P = 0.388).
Figure 3. The number of cells on each APC & APC+ Nano-HA pellet 24 h, 72 h and 1 week after seeding determined by MTT assay.

Table I. MTT data analysis and statistics of two different substrates (APC&APC +Nano-HA) for 24, 72 h and 1 week

<table>
<thead>
<tr>
<th>Time</th>
<th>silicate-based cement alone(APC)</th>
<th>Nanohydroxyapatite silicate-based cement (APC +Nano-HA)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>24.23±5.121</td>
<td>21.62±7.90</td>
<td>0.511</td>
</tr>
<tr>
<td>72 h</td>
<td>72.77±34.923</td>
<td>23.61±14.38</td>
<td>0.016</td>
</tr>
<tr>
<td>1 week</td>
<td>23.54±11.078</td>
<td>15.85±4.51</td>
<td>0.162</td>
</tr>
</tbody>
</table>

Table II. Multiple comparison analysis of different time periods of seeding

<table>
<thead>
<tr>
<th>APC(silicate-based cement alone)</th>
<th>Time</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>72 h</td>
<td>0.042</td>
</tr>
<tr>
<td>1 week</td>
<td></td>
<td>0.989</td>
</tr>
<tr>
<td>72 h</td>
<td>24 h</td>
<td>0.042</td>
</tr>
<tr>
<td>1 week</td>
<td></td>
<td>0.038</td>
</tr>
<tr>
<td>1 week</td>
<td>24 h</td>
<td>0.989</td>
</tr>
<tr>
<td>1 week</td>
<td>72 h</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Scanning Electron Microscopy (SEM) for Cellular analysis

Fibroblast cells showed good adaptation and also showed spreading cytoplasmic extensions which are essential for attachment and the proliferation of cells. A variety of cellular forms was observed. (Figure 4) The variety of cellular morphologies indicating the movement and proliferation of fibroblasts in the test materials.
Figure 3. The number of cells on each APC & APC+ Nano-HA pellet 24 h, 72 h and 1 week after seeding determined by MTT assay.

Discussion

Silicate based materials showed promising effects on apatite core formation and the combination with hydroxyapatite crystals were supposed to increase their effects. In this study, the silicate-based cement alone and its combination with Nano-HA particles with diameters of 20-25 nm are compared with each other in terms of adhesion capability with periodontal ligament fibroblast cells and silicate-based cement alone showed superior cellular proliferation. In the term of cell attachment, we found that there was no statistically significant difference between two groups at 24 h and 1 week. Although there was a significant difference between them at 72 h for the benefit of the Silicate-Based Cement alone group.

The cell population after 24 h reflected initial adhesion on the APC substrates, and a remarkable increase in cell population after 72 h indicated subsequent proliferation of the cells while there was a decline in cell population after 1 week to the level of about the initial adhesion at 24 h. MTT data analysis showed statistically significant
differences between the three time periods. In other words, there was a notable change between 24 h with 72 h and 72 h with 1 week.

On the other hand, the cell population of APC + Nano-HA substrates after 24, 72 h, and 1 week of seeding could reflect initial adhesion on the substrates with no statistically significant difference between three time periods.

Our result for APC medium is in agreement with the study conducted by Abdullah and coworkers (14). In their study cells have been found in close contact with APC (15).

Unexpected result of our study was that the cell population after 1 week of seeding in APC group decreased to the level of about initial seeding at 24 h after a significant rise at 72 h of the seeding. One probable assumption for the inhibition of cell proliferation may be the limitation of the nutrient or the surface area of the substrate necessary for the growth and proliferation in addition to the aggregation of cellular waste products in the in vitro study, while in body, blood circulation supplies the nutrients, oxygen, carbon dioxide and hormones and collects the waste materials from cells and transport to lymphatic circulation.

Fibroblast cell adhesion is important for cell division and cell proliferation (16). Typically, cell apoptosis (anoiKis) occurs when cells cannot adhere to a scaffold (17) or cannot hold their connection (18). As Okada and others have suggested, reducing the size of the nanoscale unit affects focal adhesion formation, thus influencing the adhesion and proliferation of the cell (19).

Some others have also suggested that the function of fibroblasts is suppressed on the nanostructures (20–22). Okada and others reported that the adhesion and/or proliferation of L929 mouse fibroblasts was restricted on HA nanocrystals smaller than 30 nm (19). However, there are many reports of increased cellular activity in the presence of nanostructures (20–28). Differences in these published articles may be due to the fact that cell function is sensitive to the topography and size of nanostructures. Nanoscale topographic features influence cell behaviors in terms of adhesion, morphology, migration, and proliferation (29, 30). Choi and others, suggested “the needle-like nanostructures should be useful for a biological low adhesive surface, that is, anti-adhesion or antifouling surface” (31). Gao and others (32) reported nano-topographic features to influence the functions of periodontal ligament cells. Therefore, biomaterials for nano-structured scaffolds should be carefully designed taking into account the size and characteristics of cells and substrates.

Subsequent studies should conduct further experiments to better characterize the material, including examining the expression of osteoporosis markers and cementation and comparing it with other mesenchymal stem cells isolated from dental tissues (dental pulp mesenchymal stem cells).

In general, this study revealed that both cement substrates were biocompatible although adding needle shape HA nanoparticles of 20–25 nm to the APC substrate could not enhance fibroblast cell proliferation in one week after seeding.

Conclusions

Although both Nanohydroxyapatite silicate-based cement and silicate-based cement alone are biocompatible, in terms of fibroblast cell attachment, silicate-based cement alone showed superior cellular proliferation in 72 hours. After 24 h and 1 week both minerals show the same response.

Acknowledgments

The authors appreciate the funding and support for this study provided by the Vice Chancellor of Research Center & Innovation, Shiraz University of Medical Sciences. The Scanning Electron Microscopy (SEM) images for Cellular analysis and TEM data used to support the findings of this study are included within the article.

Conflicts of interests

The authors declare no conflicts of interest.

References


**Corresponding Author**
Hooman Khorshidi,
Department of Periodontics, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran
Tell: +98-713-628-0456
Email: khorshidih@sums.ac.ir;