Effect of Collagen Chitosan Hydrogel with Injectable Platelet-Rich Fibrin on Alkaline Phosphatase Activity and Calcium Deposition An In Vitro Study on Osteoblast-like Cell Line MG63

Kent Sidharta
Gadjah Mada University

Suryono (✉️ suryonodent@mail.ugm.ac.id)
Gadjah Mada University

Kwartarini Murdiastuti
Gadjah Mada University

Mardha Ade Pritia
Gadjah Mada University

Research Article

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Abstract

Background

Periodontal tissue regeneration is one of the main goals of periodontal therapies that is consisted of 4 main components e.g., growth factor such as injectable Platelet-Rich Fibrin (i-PRF) and scaffold, using collagen chitosan hydrogel which hoped to replace bone graft because its donor risk and immunogenic responses.

Methods

Bone formation markers such as alkaline phosphatase activity, an enzyme produced by osteoblast, and calcium deposit, late marker of osteoblast mineralization were measured using ALP staining kit (SensoLyte) and Alizarin Red S staining (Merck) in 1, 7, 14, and 21 days using MG63 osteoblast and divided into 3 groups: Hydrogel i-PRF, Bone Graft i-PRF, and Control group. The result was then analyzed using Two-way ANOVA test and Post-Hoc Tukey HSD test.

Results

Hydrogel i-PRF application induced significant ALP activity in day 7 and 14 compared to Bone Graft and Control group. Calcium deposits in day 7 of Hydrogel i-PRF and Bone Graft i-PRF group were found to be significantly different to Control group. Hydrogel application still holds the highest deposit in 14 days to 21 days. These results showed the ossification capability of collagen chitosan hydrogel with i-PRF compared to bone graft application. Through PDGF and BMP content in i-PRF which are bounded to collagen fibril, acceleration of osteoblast’s proliferation and migration rate, followed by the rise of differentiation and mineralization rate of osteoblast by BMP.

Conclusion

collagen chitosan hydrogel with i-PRF application enhanced ALP activity and calcium deposit compared to bone graft with i-PRF.

1. Introduction

Periodontitis is the 11th highest prevalence disease in the world, ranging from 20 to 50% of adult suffering this disease. The main goal of periodontal therapy is total regeneration of periodontal tissues those have been loss through immune response, inflammation, or trauma with the same tissue’s structure and function. Tissue regeneration consisted of 4 main components which are stem cell, morphogen (growth factor), scaffold, and supporting environment.

Platelet concentrate has been used as growth factor due to its price, availability, and non-immunogenic property. Injectable Platelet-Rich Fibrin (i-PRF), second generation PRF with liquid consistency, have shown higher growth factor concentration, longer release, and higher regenerative potency than its earlier
In a recent study, fractionation of i-PRF to red and yellow i-PRF finds that red i-PRF has more osteogenic effect than yellow through its higher content of growth factor, cellular, and leukocyte, increasing cell proliferation and migration more rapidly than yellow i-PRF, changing the perspective of using prior i-PRF, which is yellow i-PRF.

Because of the lack of i-PRF’s mechanical strength, i-PRF quickly absorbed and degraded by tissue, decreasing its osteogenic capability. The addition of collagen chitosan hydrogel can increase i-PRF’s mechanical strength and growth factor release duration, thus increasing its osteogenic capability. Collagen fibril restricts diffusion of i-PRF’s growth factor, prolonging its effect and duration. Addition of chitosan creates hydrogen bond between collagen and chitosan making a more stable material. The combination between these two materials is often done as drug delivery system to prolong drug availability. A three-dimensional scaffold is made from hydrogel, network of polymers chain capable of holding large amount of water. With hydrogel’s porous structure, nutrient and cells migration is able to take place more conveniently. The combination of collagen chitosan hydrogel with i-PRF acts as growth factor reservoir by binding thus retard their release though this material’s complimenting effects.

Currently, bone graft is one of the gold standards of scaffold which readily available by either autograft or allograft. Autograft, which is extracted through the donor sites, has many disadvantages such as requirement of second surgery, wound at donor sites, scar tissue formation, and high-risk surgery. Allograft such as Freeze-Dried Bone Allograft (FDBA) may cause high immunogenic response and pathogen transmission to the receiver.

Osteogenic capacity of materials is measured from alkaline phosphatase activity and mineral deposition. Bone-specific alkaline phosphatase is produced by osteoblast and has become bone formation predictor through its function in increasing local inorganic phosphate and reducing extracellular pyrophosphate concentration, an inhibitor of mineral formation, thus facilitating cell mineralization. Calcium deposition is measured by staining calcium using Alizarin Red S through chelation reaction to calcium ion, resulting in red nodule. This method is considered as gold standard in calcium deposit detection that occurred in late differentiation stage.

Hence, this study aimed to measure osteogenic capacity of collagen chitosan hydrogel through its alkaline phosphatase activity and calcium deposition of MG-63-osteoblastic-like cell line.

### 2. Material and Methods

This in vitro study was approved by Research Ethical Commission of Dentistry Faculty Gadjah Mada University (163/KE/FKG-UGM/EC/2022). Cells were divided into 3 groups: Collagen-chitosan hydrogel i-PRF group, bone graft i-PRF group, and control group. FDBA was obtained through Badan Tenaga Nuklir Nasional (BATAN).

**Collagen Chitosan Hydrogel Mixture**
Chitosan was solubilized with 2% acetic acid overnight to ensure total dilution of chitosan. Collagen and HPMC powder were then added to chitosan solution and homogenized using homogenizer (*UltraTurax*) overnight. Ratio of collagen and chitosan (25/75) was acquired from a previous study which meet pH and consistency criteria. Then, pH was checked using pH meter (*Ohaus*) and brought to neutral 6.2–7 by adding enough 1M NaOH.

**Preparation of i-PRF**

From systematically healthy male volunteers with age range of 18–30 and normal thrombocyte count, 12 ml of blood was collected in plastic centrifuge tubes (*Iwaki*) after informed consent was obtained. Immediate centrifugation at 700 rpm for 3 minutes using centrifuge (*Fisher*) was done, separating the blood into 3 layers; yellow, buffy coat, and red layer. Red i-PRF was obtained through aspiration with 3 cc syringe and 18G needle on the buffy coat layer.

**Mixing of Collagen Chitosan Hydrogel i-PRF and Bone Graft i-PRF**

Both collagen chitosan hydrogel and bone graft were mixed with i-PRF with 1:1 ratio using micropipette then diluted into culture media. Control group was given hydrogel that was acquired by mixing HPMC powder into aquadest without adding other materials.

**MG63 Osteoblast Cell Culture**

MG63 osteoblast cell line (*ATCC, US*) was seeded in 96 well plate microplate with $2.5 \times 10^3$ cells/well consisting of Dulbecco’s Modified Eagle’s medium (DMEM) with 10% fetal bovine serum (*Gibco*) and maintained at 37°C in 95% humidity and 5% CO2 for overnight. Cell culture was divided 9 samples per groups: collagen chitosan hydrogel i-PRF, bone graft i-PRF, and control group and according to incubation days: 1, 7, 14, and 21 days.

**Alkaline Phosphatase Activity**

Measurement was done using Alkaline Phosphatase Staining Kit (*SensoLyte*) following its instruction manual. In the end of day 1, 7, 14, and 21, each well was washed 2 times using buffer solution provided in the kit. Adequate triton solution was added to the wells and resuspend using micropipette. The solution was then transferred to microcentrifuge tube and incubated in 4°C for 10 minutes with agitation. Centrifugation was done using refrigerated centrifuge (*Vilocity*) at 4000 rpm for 10 minutes. Approximately 50 µL of supernatant from each well was collected and mixed with 50 µL substrate solution for 30 seconds followed by incubation for 30–60 minutes in dark condition. Stop solution then added and absorbance was measured using spectrophotometer at 405 nm.

**Calcium Deposition**

Alizarin Red S Staining solution was made by mixing 2 mg Alizarin Red S powder (*Merck*) with 100 ml aquadest using vortex (*Fisher*) for 15 minutes. In day 1, 7, 14, and 21 at the end of incubation period, the
media was removed and cells were fixated using 4% formaldehyde in 4°C for 10 minutes. The cells were washed again using PBS. Staining using Alizarin Red S solution was done for 15 minutes followed by washing using deionized water for 5 times. Destaining solution consisted of 20% methanol and 10% acetic acid was added to each well then incubated for 15–20 minutes. Absorbance then measured using spectrophotometer at 405 nm.

**Statistical Analysis**

Statistical analysis was performed using SPSS v.10. For each assessment, normality and homogeneity test were done, followed by Two-Way ANOVA. After then, Post-Hoc Tukey HSD test was done to inspect significant difference between groups. Differences at p < 0.05 were considered statistically significant.

### 3. Result

**Alkaline Phosphatase Activity**

Alkaline phosphatase activity in day 1 shown no significant difference in all group sas shown in Fig. 1. This number increased significantly at day 7 in collagen chitosan hydrogel i-PRF and bone graft i-PRF group. Both collagen chitosan hydrogel i-PRF and bone graft i-PRF have significant differences to control group. Osteoblast’s ALP level in collagen chitosan hydrogel i-PRF group was significantly higher than bone graft i-PRF and control group in days 7 and 14. Alkaline phosphatase activity remained the same at day 14 compared to day 7 in collagen chitosan hydrogel i-PRF and bone graft i-PRF group, whereas control group increase significantly. In day 21, ALP level in all groups dropped significantly. ALP activity mean and standard deviation in all groups are described in Table 1.

<table>
<thead>
<tr>
<th>Incubation Days</th>
<th>n</th>
<th>Collagen Chitosan Hydrogel i-PRF</th>
<th>Bone Graft i-PRF</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>9</td>
<td>0.124 ± 0.005</td>
<td>0.118 ± 0.005</td>
<td>0.128 ± 0.008</td>
</tr>
<tr>
<td>Day 7</td>
<td>9</td>
<td>0.159 ± 0.014</td>
<td>0.140 ± 0.007</td>
<td>0.116 ± 0.005</td>
</tr>
<tr>
<td>Day 14</td>
<td>9</td>
<td>0.160 ± 0.011</td>
<td>0.140 ± 0.007</td>
<td>0.142 ± 0.010</td>
</tr>
<tr>
<td>Day 21</td>
<td>9</td>
<td>0.121 ± 0.009</td>
<td>0.114 ± 0.005</td>
<td>0.112 ± 0.007</td>
</tr>
</tbody>
</table>

**Calcium Deposition**

In day 1, no significant difference found in all groups as shown in Fig. 2 with calcium deposition mean and standard deviation are described in Table 2. All groups increased significantly at day 7 with i-PRF-treated groups differed significantly with control group. Mineral deposition in collagen chitosan hydrogel increased significantly compared to control group in day 7, 14, and 21 also to bone graft group in day 14
and 21. Bone graft i-PRF group calcium deposition decreased significantly at day 14. Calcium nodule was also found in one of the collagen chitosan hydrogel group’s well at day 21 pictured in Fig. 3.

Table 2
Mean and Standard Deviation of Osteoblast Alkaline Calcium Deposition

<table>
<thead>
<tr>
<th>Incubation Days</th>
<th>n</th>
<th>Collagen Chitosan Hydrogel i-PRF</th>
<th>Bone Graft i-PRF</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>9</td>
<td>0.049 ± 0.003</td>
<td>0.050 ± 0.002</td>
<td>0.048 ± 0.004</td>
</tr>
<tr>
<td>Day 7</td>
<td>9</td>
<td>0.117 ± 0.004</td>
<td>0.120 ± 0.006</td>
<td>0.105 ± 0.004</td>
</tr>
<tr>
<td>Day 14</td>
<td>9</td>
<td>0.115 ± 0.005</td>
<td>0.097 ± 0.006</td>
<td>0.087 ± 0.006</td>
</tr>
<tr>
<td>Day 21</td>
<td>9</td>
<td>0.109 ± 0.007</td>
<td>0.099 ± 0.005</td>
<td>0.093 ± 0.005</td>
</tr>
</tbody>
</table>

4. Discussion

Regenerative periodontal therapy designed to regenerate periodontal tissues use multiple materials each with their own purposes with the aim to accelerate certain cells. Regenerating bone through adding scaffold and applying membrane are some of periodontal treatments those rely on osteogenic material. Bone graft, specifically FDBA, is one of the gold standards of scaffold used in periodontal treatment. Price, availability, and immunogenic response may become the deciding factor of their usage as osteogenic material. A newer, cheaper, and superior bone regeneration materials is needed to help dentists improving patient’s periodontal status and quality of life.

Collagen chitosan hydrogel is one of bio scaffold materials consisted of osteogenic components. Collagen is considered as ideal scaffold material because its low antigenicity and high cytocompatibility.\(^{13}\) It can increase bone density by inducing osteoblast activity and bone matrix maturation by increasing ALP activity, osteogenic mRNA, and protein expression.\(^{14}\) Chitosan hydrogel has been also used in drug delivery system from oral, ocular, and nasal as wound healing accelerator and tissue engineering material through its porous structure.\(^{15}\)

Alkaline phosphatase is expressed in early development of bone and decreases as the development progresses. This enzyme is produced by osteoblast to promote bone formation through the hydrolysis of pyrophosphate (PPi), a mineralization inhibitor. Pyrophosphate acts as bone mineralization regulator by inhibiting crystallization of calcium ion and Pi to form hydroxyapatite. It can also bind strongly to hydroxyapatite, preventing further crystal growth. The abundance of Pi ion enhances the production of hydroxyapatite, hence increasing bone formation.\(^{16}\)

Greater number of cell (leukocyte, platelet, erythrocyte) and PDGF found in red i-PRF results in higher proliferation and migration of PDLSC. The earlier upper yellowish layer described as i-PRF, found to be inferior compared to the red i-PRF proposed by the researchers.\(^{5}\) Among the growth factors contained in i-PRF, the main groups of growth factors associated with bone regeneration are VEGF, IGF, TGF-\(\beta\), PDGF,
and BMP, presented great potentials in bone healing and osteogenesis through regulating cell behavior, including recruitment, migration, adhesion, proliferation, and differentiation.\textsuperscript{17} Growth factor delivery strategies, specifically hydrogel, for sustained release and stability of GF is needed to promote bone healing.

In our study, we observed that collagen chitosan hydrogel i-PRF increased ALP activity and calcium deposition compared to bone graft i-PRF and control groups at day 7, 14, and 21 in osteoblast. In day 1, osteoblast ALP level shown no significant difference in all groups. This result demonstrates alkaline phosphatase as early osteoblastic marker increasing after 7 days following the proliferation rate. These numbers increased significantly at day 7 in collagen chitosan hydrogel i-PRF and bone graft i-PRF group, showing significant difference to control group. These shown the effect of i-PRF as growth factor for osteoblast in increasing its early proliferative marker. Collagen chitosan hydrogel i-PRF group ALP activity was also significantly different with bone graft group which demonstrates the advantages of collagen chitosan hydrogel over bone graft as scaffold material. Through chitosan and hydrogel porous structure, osteoblast interaction with other active agent, such as growth factor, can take place more conveniently through hydrogel's porosity.

Growth factor contained in i-PRF such as Platelet-Derived Growth Factor (PDGF), Bone Morphogenetic Protein (BMP), Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor Beta 1 (TGF-\(\beta\)1), Insulin-Like Growth Factor 1 (IGF-1), and Epidermal Growth Factor (EGF) can interact with osteoblast. Previous study shown effective therapy in bone regeneration by using growth factor combination, PDGF and BMP-6. PDGF’s role in osteoblast matrix deposition increases its proliferative and migration rate whereas BMP-6 can accelerate differentiation and mineralization of osteoblast.\textsuperscript{18} In another study, endothelial and osteoblast coculture exhibited increased VEGF and BMP-2 level which control osteoblast ALP expression and mineralization.\textsuperscript{19}

In day 14, ALP activity in collagen chitosan hydrogel i-PRF and bone graft i-PRF group stays relatively the same with day 7 which shows i-PRF’s ability in maintaining ALP expression until day 14. ALP level may be peaked between day 7 and day 14 when collagen matrix deposition reaches its higher point, ending the proliferative period and starting mineralization stage.\textsuperscript{20} However, collagen chitosan hydrogel i-PRF group still shown significant difference with all groups. Through collagen fibril inside the hydrogel, growth factor molecules those directly loaded into can diffuse slower through collagen little pores. Direct loading of growth factor into collagen mainly has rapid burst release profile, but PDGF-BB has sustained release profile parallel to biodegradation of collagen.\textsuperscript{21}

All groups ALP activity dropped significantly at day 21 showing no significant difference between groups. This decrease shown no significant difference against day 1, showing ALP level in all groups reverted to day 1. This happened as osteoblast enter to the end of mineralization stage, showing low early differentiation marker expression including ALP. In the late differentiation stage of osteoblast, many osteoblasts died due to well’s confluence and maturation, leaving calcium nodule inside the well. These
calcium ions reside intracellularly as granule in mitochondria or inside matrix vesicle and promote extracellular matrix mineralization through calcium and phosphate storage.\textsuperscript{22}

Calcium deposition in day 1 shown no significant difference in all groups. This late differentiation marker is not yet to emerge at the early stage of osteoblast. This number went up significantly at day 7 compared to day 1. Both collagen chitosan hydrogel i-PRF and bone graft i-PRF group calcium deposition were found to be significantly higher control group, as seen as ALP activity in day 7. The osteogenic capability of i-PRF causes the increasing number of mineral depositions in both groups. This shown the effect of growth factor contained in i-PRF effect on accelerating osteoblast mineralization to begin before control group mineralization started. Hence, higher calcium ion level found in i-PRF treated groups.

Day 14 shown calcium deposition of collagen chitosan hydrogel staying relatively the same with day 7 with the highest level of calcium between all groups. The superiority of collagen chitosan hydrogel over bone graft is shown through this result. Bone graft i-PRF group's calcium deposition decreased significantly at day 14 demonstrating bone graft failed to maintain mineral deposition in the late differentiation stage of osteoblast. The calcium deposition stays nearly the same at day 21 with collagen chitosan hydrogel still holds the highest calcium content, showing significant difference with other groups. This shown sustain released of growth factor by collagen chitosan hydrogel until late differentiation stage of osteoblast ended. Further studies about growth factor release profile of red i-PRF and collagen chitosan hydrogel is needed to elucidate this biomaterial scaffold role in periodontal tissue regeneration.

The use of 2D cell culture in this research has several disadvantages such as assessments of ALP activity and calcium deposition was limited to attached cell only, disregarding the cell inside of the hydrogel. This happened because the cell inside of the hydrogel get washed away as the requirement of the assay used for 2D cell culture. Hydrogel function as growth factor and cell reservoir cannot work properly, thus the result may be lessened than the actual bone formation. Hence, 3D cell culture is recommended as this study focus on scaffold and the length of this study makes 2D cell culture less effective. Through 3D cell culture, monitoring viability; proliferation; differentiation; and function of osteoblast can be done thoroughly with higher in vivo relevance.\textsuperscript{23}

5. Conclusion

Hence, within the limitations of this study, it can be concluded that application of collagen chitosan hydrogel with i-PRF causes increased alkaline phosphatase activity and mineralization at day 1, 7, 14, and 21 in MG-63 osteoblast-like cells. Application of collagen chitosan hydrogel with i-PRF may provide early and improved bone formation better than FDBA.

Declarations

\textit{Ethics Approval}
Ethic was approved by Komisi Etik Fakultas Kedokteran Gigi dan RSGM Prof Soedomo, Gadjah Mada University number 163/KE/FKG-UGM/EC/2022. All methods were carried out in accordance with relevant guidelines and regulations.

Consent for Publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interest

The authors declare that they have no competing interests.

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Authors’ contributions

KS performed the measurement ALP and calcium deposit, was a major contributor to the writing of this manuscript. MAP was a major contributor of this manuscript and made all of the figures and tables provided in this research. S was a major contributor to the writing of this manuscript. KM analyzed and interpreted the data obtained in this research, was a major contributor to the writing of this manuscript. All authors read and approved the final manuscript.

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References


Figures

[Graph showing Alkaline Phosphatase Activity]

Figure 1

Alkaline Phosphatase Activity in Day 1, 7, 14, and 21
Figure 2

Calcium Deposition in Day 1, 7, 14, and 21

Figure 3

Calcium Nodule Found in One of Collagen Chitosan Hydrogel i-PRF Group’s Well