ALP gene expression in cDNA samples from bone tissue engineering using a HA/TCP/Chitosan scaffold

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Abstract. This study examined the potential use of hydroxyapatite (HA)/tricalcium phosphate (TCP)/Chitosan as a bone tissue engineering scaffold. The potential for using HA/TCP/chitosan as a scaffold was analyzed by measuring expression of the ALP osteogenic gene in cDNA from bone biopsies from four Macaque nemestrina. Experimental conditions included control (untreated), treatment with HA/TCP 70:30, HA/TCP 50:50, and HA/TCP/chitosan. cDNA samples were measured quantitively with Real-Time PCR (qPCR) and semi-quantitively by gel electrophoresis. There were no significant differences in ALP gene expression between treatment subjects after two weeks, but the HA/TCP/chitosan treatment gave the highest level of expression after four weeks. The scaffold using the HA/TCP/chitosan combination induced a higher level of expression of the osteogenic gene ALP than did scaffold without chitosan.

1. Introduction

Bone is a living tissue with a rich blood supply and the specific capacity to regenerate. Bone serves as a structural scaffold where the muscles attach and has a role in their contraction, supports vital organs including the brain and spinal cord, is a major source of calcium and phosphate, and provides a micro-environment suitable for hematopoietic cell differentiation [1]. Pathological conditions that damage vital bone components can cause a decrease in at least one of the functions of the bone and can cause bone defects [2]. In small bone defects, the bone can regenerate spontaneously or with minimal intervention. However, bones often cannot repair large defects on their own, due to the amount of damage and the slow regeneration process. Examples of large defects would include injuries resulting from car accidents, congenital cleft palate, bone lesions due to cancer removal, or periodontal abnormalities [1].

Bone defects can be treated by various methods, such as autografts, allografts, and metal implants. The autograft method involves placing osteogenic, osteoinductive, and osteoconductive components in the defect area without triggering a host immune response. However, the quantity of bone produced is limited, and extensive surgery may be required, which can cause morbidity, pain, or infection in the treated area [1]. Moreover, this approach is limited to large defects [3]. The allograft method can overcome the problem of bone quantity, but has less osteoinductivity, can trigger host immune responses, and allows disease transmission. The metal implant method is a suitable alternative treatment for some cases. However, it requires a second operation to remove the metal since the metal cannot be degraded after the implantation and may release toxic ions that trigger a host immune response and subsequent infection.
Tissue engineering is a multidisciplinary field that aims to develop techniques to repair or regenerate damaged tissues and their functions. Tissue engineering techniques provide potential alternatives to bone grafts, with the advantages of an unlimited supply and no disease transmission [1,4]. These techniques have been developed to repair large bone defects that exceed the body's ability to regenerate and cannot be handled by bone grafts alone [5,6], as in the case of a non-union fracture [6].

Tissue engineering techniques require three main components, namely a cell, scaffold material, and a growth factor/signal. Scaffold is a matrix that can facilitate the ability of cells to attach, proliferate, and differentiate to form desired tissue [5]. An ideal scaffold is non-toxic, non-allergenic, non-irritating, non-genotoxic, non-carcinogenic, biocompatible, biodegradable, porous, and can mimic extracellular liquid [7].

Selection of appropriate biomaterials for scaffold is important, because the characteristics of this material could potentially affect cell biological activity [8]. Compounds that are most often used for scaffolding in tissue engineering of bone are hydroxyapatite (HA) and tricalcium phosphate (TCP) [1]. The lack of mineralization of calcium and phosphate during the natural bone regeneration phase can reduce the mechanical strength of tissue, so calcium phosphate biomaterials are an appropriate choice for use in bone tissue engineering [9]. HA and TCP are major components of the natural bone matrix, and thus are highly biocompatible [1,10]. HA and TCP have osteoconductive properties, with the ability to bind to bone and soft tissue, and to stimulate bone growth [11]. Although HA and TCP have suitable properties for bone tissue engineering, they are fragile, rigid (hard to adjust to a specific form required as a bone substitute, and have a low mechanical stability [1,12]. The degradation rate of HA/TCP is highly unpredictable, which can result in increased calcium and phosphate concentrations in the blood [1]. In addition to HA and TCP, chitosan is also widely used as scaffold in tissue engineering. Chitosan is a natural biopolymer derived from chitin obtained from the shells of crustaceans. It has ideal scaffold properties, being biocompatible, biodegradable, non-toxic, non-immunogenic, and non-carcinogenic [13]. Chitosan has many active groups, including amino and hydroxyl groups that allow modifications to improve its physical and chemical properties [14], and it can bind covalently to biomolecules including fructose, galactose, and RGD peptides. Composite chitosan can be made by combining chitosan with a bioactive material, such as calcium phosphate particles, to support osteogenesis [7].

Several studies on the use of HA/TCP as a tissue engineering scaffold have been done. In a study comparing the use of HA/TCP to pure HA transplanted to the dorsum of a rat, minimal bone formation was seen on the HA transplantation relative to HA/TCP [9]. Another study, comparing HA/TCP to TCP in osteogenic activity, showed substantial layers of bone tissue with abundant extracellular matrix on the scaffold implantation of HA/TCP, but the TCP scaffold had little new bone tissue [11]. These studies demonstrated that HA/TCP has better osteogenic potential than pure HA scaffold or pure TCP scaffold. The use of a composite of chitosan and HA (HA/chitosan) in bone tissue engineering has also been studied. The HA/chitosan had increased osteoconductivity, bioactivity, bone-binding ability, and elasticity over HA [12]. However, bone tissue engineering using chitosan scaffold combined with HA/TCP had not been examined. The combination of HA/TCP/chitosan was hypothesized to reduce the rigid nature of HA/TCP, improve the mechanical properties of chitosan, and generate more ideal scaffold properties in supporting osteogenic differentiation. Osteogenic differentiation can be characterized by the markers of bone formation [15]. Alkaline phosphatase (ALP) is an enzyme that is involved in the formation of osteoid and bone mineralization, and is expressed at the initial phase of bone establishment [16,17]. Therefore, ALP is often used as a bone formation marker [18]. Since the potential of adding chitosan material to a HA/TCP scaffold is not known, the present study compared the gene expression of ALP in samples from subjects that had been treated with HA/TCP 70:30, HA/TCP 50:50, or HA/TCP/chitosan.

2. Materials and Methods
The experiments were performed in the Oral Biology Laboratorium at Faculty of Dentistry Universitas Indonesia, Salemba, between September and November, 2015. cDNA was prepared from bone biopsy
samples from *Macaque nemestrina*. The animals were designated as control or treatment subjects. Bone biopsies were taken on day 0 (baseline), and two weeks and four weeks after transplantation. The control received no treatment, while treatment subjects each received a combination of scaffold material, either HA/TCP 50:50, HA/TCP 70:30, or HA/TCP/chitosan.

Real-Time PCR (qPCR) was used to quantitatively measure the expression of *ALP* in samples from the baseline, second week, and fourth week. Analyses of the qPCR products were done using *StepOne v.2.3* (Applied Biosystems, Foster City, CA, USA). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous reference to normalize *ALP* expression. Primers used for the qPCR reaction are shown in Table 1.

| Gene   | Primers                                      |
|--------|----------------------------------------------|
| *ALP*  | Forward 5'-GCTTCAAACCGAGATACAAGCA-3'         |
|        | Reverse 5'-GCTCGAAGAGACCCAATAGGTAGT-3'       |
| *GAPDH*| Forward 5'-ATGGGGAAGGTGAAGGTCG-3'            |
|        | Reverse 5'-TAAAAGCAGCCCTGGTGACC-3'           |

Table 2 shows the PCR reaction mix.

| Materials                  | Volume |
|----------------------------|--------|
| Reagent (DNA Polymerase, dNTP, optimized buffer, dye, passive reference) | 7.5 µl |
| Primer Forward (6 µM)      | 0.75 µl |
| Primer Reverse (6 µM)      | 0.75 µl |
| H2O, nuclease free         | 1 µl   |
| Sample cDNA                | 5 µl   |
| Total                      | 15 µl  |

Relative gene expression was determined using the comparative Ct method, and included the Livak and Schmittgen equation (2001), which is $2^{-\Delta\Delta Ct}$. $\Delta\Delta Ct$ is obtained through this calculation:

1. Target gene normalization using reference gene, by subtracting target’s Ct gene (*ALP*) with Ct reference gene (*GAPDH*) → $\Delta Ct$ was acquired

2. Subtracting test $\Delta Ct$ from control $\Delta Ct$ (control or baseline) → $\Delta\Delta Ct$ is obtained.

The qPCR products were analyzed using gel electrophoresis (1.5% agarose) and visualized using Gel Documentation (Quantity One Universal Hood; Bio-Rad, Hercules, CA, USA). The electrophoresis results were analyzed by comparing a 100 base pair (bp) DNA ladder arrangement to the test cDNA. This was done to confirm that the gene that was amplified was the gene of interest. The intensity of each band obtained from electrophoresis was analyzed to determine the level of gene amplification. The intensity was determined through a semi-quantitative calculation using the Image J software.
semi-quantitative calculation was performed independently by two examiners to measure data consistency and reliability. This was followed by a kappa statistic test, to confirm that there were no significant differences between the examiners’ results. If the statistic test showed no significant differences, then the average of the two examiners’ data was used.

In this study, the statistical analysis was done using IBM SPSS Statistic Version 22. Statistic test is done using 0.05 significance (p = 0.05) and a confidence level of 95%. A normality test was done on the samples, using the Shapiro-Wilk equation. After obtaining a normalized data distribution, a T-test statistical analysis was performed to compare the mean of the two groups. For a small sample size, a nonparametric test is used to compare both groups using the Kruskal-Wallis H test (more than two groups) and Mann-Whitney U test (two groups).

3. Results and Discussion

3.1 Results

Figure 1. Relative expression of ALP after two weeks compared to baseline

Based on the Kruskal-Wallis H test, there was no significant difference of expression of ALP in any treatment subject after two weeks relative to baseline. p = 0.14 (p ≥ 0.05).

Figure 2. Relative expression of ALP after two weeks compared to control
Based on the Kruskal-Wallis H test, there was no significant difference of expression of ALP in any treatment subject (HA/TCP 70:30, HA/TCP 50:50, HA/TCP/chitosan) relative to the control subject at week two. $p = 0.14 \ (p \geq 0.05)$.

**Figure 3.** Expression of ALP in control and treatment subject HA/TCP 70:30 after two weeks

Based on the Mann-Whitney U test, there was no significant difference of expression of ALP between the treatment subject HA/TCP 70:30 and the control subject after two weeks. $p = 0.121 \ (p \geq 0.05)$.

**Figure 4.** Expression of ALP in control and treatment subject HA/TCP 50:50 after two weeks.

Based on the Mann-Whitney U test there was no significant difference of expression of ALP between the control and treatment subject HA/TCP 50:50 after two weeks. $p = 0.083 \ (p \geq 0.05)$. 


Based on the T-test, there was no significant difference of expression of ALP between the control and treatment subject HA/TCP/Chitosan after two weeks. $p = 0.105$ ($p \geq 0.05$).

**Figure 5.** Expression ALP in control and treatment subject HA/TCP/Chitosan after two weeks

Based on the T-test, there was a significant difference in mean relative expression of ALP between the HA/TCP 50:50 treatment subject and the HA/TCP/chitosan treatment subject after four weeks. $p = 0.027$ ($p \leq 0.05$).

**Figure 6.** Expression of ALP in the HA/TCP 50:50 treatment subject and the HA/TCP/Chitosan treatment subject
Figure 7. Gel electrophoresis of PCR products

Table 3. ALP PCR Band Intensity

| Scaffold          | ALP Band Intensity       |
|------------------|--------------------------|
| HA/TCP 70:30     | 3619.73 ± 50.53 pixel    |
| HA/TCP 50:50     | 3565.49 ± 1086.11 pixel  |
| HA/TCP/Chitosan  | 3132.73 ± 1854.89 pixel  |
| NTC              | 61.314 pixel             |

Table 4. GAPDH PCR Band Intensity

| Scaffold          | GAPDH Band Intensity     |
|------------------|--------------------------|
| HA/TCP 70:30     | 2812.96 ± 1078.52 pixel  |
| HA/TCP 50:50     | 5584.66 ± 4141.54 pixel  |
| HA/TCP/Chitosan  | 2875.72 ± 214.51 pixel   |
| NTC              | 63.364 pixel             |

Based on the Kruskal-Wallis H test, there was no significant difference of GAPDH band intensity between the treatment subjects (HA/TCP 70:30, HA/TCP 50:50, HA/TCP/chitosan).

3.2 Discussion

In this study, samples of cDNAs synthesized from RNAs were used to determine gene expression levels. The cDNAs were standardized to equalize the concentration of each sample before performing qPCR. Standardization was performed on cDNA rather than on RNA because DNA is more stable than RNA [19]. After standardization, qPCR was performed to measure expression of ALP in each cDNA sample.

Two researchers independently performed qPCR on the samples in duplicate, resulting in four PCR products for each sample. However, some data from the qPCR were excluded due to the existence of primer-dimers and a high standard deviation on several measurements of the same sample. Primer-dimers are nonspecific products that are amplified by PCR when two PCR primers bind to one another rather than to the target DNA. The presence of primer-dimers can reduce the efficiency of PCR, thereby affecting the fluorescent signal in a qPCR reaction, lowering the sensitivity, and obscuring analysis of the results. Primer-dimers may be identified through the melting curve contained on test
results of qPCR, which is seen as an additional peak next to the amplified product’s peak. In this study, some samples had a melting curve with a peak greater than 1, indicating the presence of primer-dimers in the product. Data from those samples were excluded to minimize qPCR analysis error [20]. There were also high standard deviations on some measurements of Ct in one sample, creating a diminishing reliability of the measurements. Data from that sample were also excluded.

cDNAs that were tested using qPCR were from the untreated control and subjects that had been treated with the scaffold compounds HA/TCP 70:30, HA/TCP 50:50, or HA/TCP/chitosan. Samples were taken on day 0, and at week 2 and week 4. The addition of chitosan to the scaffold material was expected to support osteogenesis, as measured by the expression of ALP.

The first test compared the control subject and treated subjects at baseline. cDNA samples were generated from bone biopsies taken before the treated subjects were given HA/TCP 70:30, HA/TCP 50:50, or HA/TCP/chitosan. As expected, the differences in expression of ALP between each of the treated subjects and the control subject were not statistically significant. Statistical analysis also showed that there were no significant differences in ALP expression after two weeks of treatment.

Statistical tests on the relative levels of expression of ALP between different treatment subjects after two weeks showed no significant differences. However, after two weeks the expression of ALP in the subject treated with HA/TCP/chitosan was higher than that in the subject treated with HA/TCP 50:50, and the subject treated with HA/TCP 50:50 had a higher level of ALP expression than did the subject treated with HA/TCP 70:30. These observations were consistent with several previous studies, where the use of a scaffold combination of chitosan and HA/TCP/chitosan resulted in ALP expression higher than that of chitosan alone [21,22]. This suggests that chitosan may be useful as a scaffold in supporting osteogenesis when combined with other materials, in particular HA and TCP, based on the visible increase in ALP expression compared to other treatments.

A baseline comparison of ALP expression in control and treated subjects showed no statistically significant differences. However, after two weeks, ALP expression in the subjects treated with HA/TCP 50:50 and HA/TCP/chitosan was higher than that in the control subject. This suggests that there was increased osteoblast activity after two weeks of treatment, although it was not statistically significant. This is consistent with research conducted by Solomon and Wongwitwichot, showing the scaffold combination of HA/TCP can support bone formation better than HA or TCP alone [9,11]. In contrast, the subject treated with HA/TCP 70:30 had a trend of decreasing ALP expression. This may be due to the difficulty of controlling the rate of degradation of HA due to its instability [10].

A comparison of the treated subjects at four weeks showed a significant increase in ALP expression in the subject treated with HA/TCP/chitosan relative to the other treated subjects. This is consistent with the findings of Venkatesan [23], that the composite HA/chitosan had osteoconductive, osteoinductive, and osteogenic properties, making it potentially useful in bone engineering processes.

After qPCR testing, gel electrophoresis was performed to ensure that the amplified gene was the desired gene. Using 100 bp ladder as a standard, the PCR product of the ALP gene was identified as a band at about 60 bp. This was consistent with the primers selected to amplify ALP. The electrophoresis results were also used to confirm that the calculations of gene expression levels from the qPCR data were directly proportional to the intensity of the resulting electrophoresis bands. The gel electrophoresis was analyzed using Image J software, to measure the intensity of the amplified bands. The intensities of the ALP bands were not significantly different between the treated subjects.

4. Conclusion
This study demonstrated a trend of increased ALP expression in subjects treated with test combinations of scaffold materials compared with the untreated control subject, as well as increased expression after two weeks in the subject treated with HA/TCP/chitosan compared with the subjects treated with HA/TCP 70:30 or HA/TCP 50:50. These trends did not reach a level of statistical significance. However, after four weeks, the subject treated with HA/TCP/chitosan had the highest of levels of ALP expression for all scaffold treatments. It was concluded that scaffold using a chitosan combination may produce increased expression of the osteogenic gene ALP compared to its
expression with commonly used scaffold materials. Future studies on the use of chitosan in scaffolding material will add to the field of bone tissue engineering.

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