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# Effect of combined application of bFGF and inorganic polyphosphate on bioactivities of osteoblasts and initial bone regeneration

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**Abstract**

Basic fibroblast growth factor (bFGF) and inorganic polyphosphate (poly(P)) have been recognized as therapeutic agents that enhance bone regeneration. It has also been shown that poly(P) may enhance the mitogenic activity of bFGF. The purpose of this study is to evaluate the combined effect of bFGF and poly(P) on bioactivities of osteoblasts and initial bone regeneration in vitro and in vivo. MC3T3-E1 cells were treated with bFGF, poly(P) or bFGF+poly(P), then subjected to cell proliferation assay, alkaline phosphatase (ALP) activity measurement, quantitative real-time reverse transcription-polymerase chain reaction and Alizarin S Red staining. In an in vivo study, bFGF-, poly(P)- and bFGF+poly(P)-modified interconnected porous hydroxyapatite (IPHA) complexes were fabricated, and placed into the femurs of rabbits to evaluate new bone formation histologically and histomorphometrically. The highest enhancement of cell proliferation were observed in those treated with bFGF+poly(P) on days 5 and 7. Cells treated with bFGF+poly(P) also exhibited increased ALP activity on days 5 and 10, up-regulated mRNA levels of osteocalcin and osteopontin, and enhanced calcification when compared to the non-treated cells. In vivo, the highest bone formation ratio was observed in bFGF+poly(P)-modified IPHA complexes. This study indicated that co-application of bFGF and poly(P) may provide enhanced bone formation by modulating cell proliferation and the mineralization process. It is anticipated that a combined application of bFGF and poly(P) can provide a novel method for bone regeneration in clinical use.


*Keywords:* Basic fibroblast growth factor; Polyphosphate; Cell proliferation; Osteogenic differentiation; Bone regeneration

## 1. Introduction

The repair of bone defects is of key interest in dental implant treatment and in orthopedics. Although bone graft substitutes, including autogenous bone, allogeneic bone, xenogeneic bone and some ceramics, such as hydroxyapatite (HA) and tricalcium phosphate, are currently available options for defect repair, none of those have been demonstrated to be fully satisfactory [1].

Most recently, a number of therapeutic agents, such as the basic fibroblast growth factor (bFGF), polyphosphate (poly(P)), platelet-derived growth factor and bone morphogenetic protein, have been used with tissue engineered techniques to enhance the bone regeneration [2]. Among these agents, bFGF, an endogenous polypeptide growth factor, is characterized by its affinity for the glycosaminoglycan heparin-binding sites on cells and has been shown to be a potent mitogen for a variety of mesenchymal cells [3,4]. bFGF may modulate bone formation through regulating the proliferation and differentiation of cells of osteoblastic

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lineage [5,6], and its ability to accelerate bone regeneration and fracture-healing has been widely reported [7–11]. However, bFGF is an unstable protein with a short half-life value, and may inhibit collagen type I (COL-1) gene expression and alkaline phosphatase (ALP) activity of osteoblasts [6,12].

Recently, Shiba et al. [13] reported that poly(P) may stabilize bFGF and facilitate attachment to its receptors, thereby enhancing its mitogenic activity. These data suggest that poly(P) may function as a control for bFGF activity. Poly(P) is composed of linear polymers of many tens or hundreds of orthophosphate residues linked by high-energy phosphoanhydride bonds and has been found in a wide range of organisms, including bacteria, fungi, algae, mosses, insects and protozoa, as well as in the tissues of higher plants and animals, including humans [14–16]. The involvement of poly(P) in apoptosis and in modulation of the mineralization process in bone tissue has already been suggested [17,18]. Poly(P) has been shown to up-regulate the expression of osteogenic genes and to increase the ALP activity in osteoblast-like MC3T3-E1 cells, human dental pulp cells and mesenchymal stem cells [19–21]. Based on these recent findings concerning the relationship of bFGF and poly(P) and osteogenesis, the combined application of these two factors could be expected to synergistically enhance bone regeneration.

This study was designed to evaluate effects of the combined application of bFGF and poly(P) on MC3T3-E1 cell proliferation, ALP activity, osteogenic marker genes expression and cell calcification, and to test the initial bone regeneration in bFGF+poly(P)-modified interconnected porous hydroxyapatite (IPHA) complexes placed into the femurs of New Zealand rabbits.

## 2. Materials and methods

### 2.1. Materials

Poly(P) (a sodium salt with an average chain length of 60 phosphate residues, Regenetiss Inc., Japan) was selected and its concentration is provided in terms of phosphate residues. bFGF was purchased from Sigma (St. Louis, MO, USA) for in vitro experiments and from Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan) for in vivo experiments. MC3T3-E1 osteoblast-like cells were obtained from Riken Cell Bank (Tsukuba, Japan). Eagle's minimum essential medium alpha modification (α-MEM), L-glutamine, fetal bovine serum (FBS) and formalin were obtained from Sigma (St. Louis, MO, USA). Penicillin–streptomycin mix was purchased from Invitrogen Life Technologies (Baltimore, MD, USA).

### 2.2. Cell proliferation assay

MC3T3-E1 osteoblast-like cells were seeded on 96-well culture plates at a density of 3000 cells well$^{-1}$ and incubated in normal culture medium (α-MEM with 5% FBS and 1% penicillin–streptomycin). Cells were incubated for approximately 8 h for initial attachment. After attachment, the culture medium was aspirated and then cells were treated with the normal culture medium containing 1 ng ml$^{-1}$ bFGF, 1 mM poly(P), 1 ng ml$^{-1}$ bFGF + 1 mM poly(P) or normal culture medium containing neither bFGF nor poly(P) as a control. Culture medium was changed every 4 days. The relative number of viable cells in each well at each experimental time point was determined using Cell Proliferation Reagent WST-1 (Roche, Indianapolis, IN, USA). Briefly, 10 μl of reagent was added to each well, including three wells containing only medium for background subtraction. After incubation at 37 °C for 1 h, the absorbance at 450 nm was measured using a microplate reader (MPR A4i, Tosoh Corporation, Tokyo, Japan).

### 2.3. Measurement of ALP activity

Cells were seeded on 24-well culture plates at a density of $2 \times 10^4$ cells well$^{-1}$. At each time points, cells were washed three times with Tris buffer (10 mM Tris–HCl, pH 7.2) and lysed in 0.2 vol.% Triton X-100 in the Tris buffer for 10 min at room temperature. The cell suspension was then transferred to a centrifuge tube, treated by ultrasonication and centrifuged (7200 g for 10 min, 4 °C). The ALP activities of the samples were determined by a colorimetric assay using an ALP reagent containing *p*-nitrophenyl phosphate (*p*-NPP) as substrate (BioAssay, Hayward, CA, USA). The absorbance of *p*-nitrophenol formed by the hydrolysis of *p*-NPP, catalyzed by ALP, was measured at 405 nm every 20 min. The total protein content was determined using a commercial Micro BCA protein assay kit (Pierce, Rockford, IL, USA). The ALP activity was then normalized to its protein concentration and expressed as units per gram of protein.

### 2.4. RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted by QIAshredder and Rneasy Mini kits (Qiagen, Valencia, CA, USA) strictly according to the manufacturer's instructions. The concentration and purity of freshly isolated RNA was measured at 260 nm using a spectrophotometer and with A260/280 ratio, respectively. The first-strand cDNA was synthesized from 1 μg RNA with TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA), and used for quantitative real-time PCR. The expression levels of genes of interest were quantified with an ABI Prism 7700 Sequence Detection System, TaqMan® Universal PCR Master Mix and pre-developed probes (Part No.: osteocalcin (OCN), Mm00649782_gH; osteopontin (OPN), Mm00436767_m1; collagen type I (COL-1), Mm00801666_g1). The program used was 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative expression levels of genes were analyzed using the $2^{-\Delta\Delta C_t}$ method [22] by normalizing with 18S rRNA control (Part No.

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