Research Report

# *In vitro* evaluation of FGF-2 on osteoblast cell seeded in nano chitosan-carbonate apatite chitosan scaffold

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**Abstract:** **Background:** Scaffold is a medium for extracellular matrix synthesis and Becomes a place for attachment and growth of new cells. Some polymeric materials, the which have been developed in tissue engineering are chitosan. Chitosan is one of the best materials used in tissue engineering due to its biodegradable, biocompability, anti-bacterial properties, for wound healing and bioadded characters. The carbonate apatite material is used as a biocompatible material for medical purposes. Carbonate apatite Increased material the chemical bonds between biomaterials and bone tissue that can Affect bone formation. The nanostructured biomaterials Significantly Enhance cell functioning leading to Increased osteoinductivity and oseointegrativity. So based on the explanation and understanding of the mechanisms of the relationship between the bone regeneration process and the nano chitosan-carbonate apatite scaffold, it is important to improve the method for the next generation of scaffolds that can demonstrate bone efficiency and regeneration in vitro. **Objective:** To Determine the Increased expression of FGF-2 on osteoblast cell planting in nano chitosan-carbonate apatite scaffold and the results of this study are expected to be used as a selection method of bone graft using nano-chitosan scaffold. **Methods:** Laboratory experimental study with post test only group design on seven samples, the osteoblast cells planted on chitosan nano-carbonate apatite scaffold with observation time on day 3, day 5, and day 7 and Increased expression of FGF- 2 osteoblast cells were measured by Immunohistochemistry (IHC) method. The results were Analyzed by statistical test of multiple comparison test of Post Hoc and Tukey test. **Result:** The results Showed a significant difference ($P < 0.05$) between treatment groups in the observation time on day 3, 5 and 7. **Conclusion:** The experiment has shown that the increase is of FGF-2 expression in osteoblast cells planted in chitosan nano-carbonate apatite scaffold has Increased in observation time on day 3, 5 and 7.

## INTRODUCTION

Based on the data Riskesdas (2007), that the rate of loss of all teeth in Indonesia is estimated to reach 1.6% of the population of Indonesia. In general, the prevalence of Indonesian people who have to wear a prosthesis in the form of loose dentures or artificial teeth fixed as much as 4.5% of the total population. The absence of teeth in the arch can occur due to congenital abnormalities, as a result of a disease, dental caries and periodontal damage and post traumatic wounds gigi1 extraction. Tooth loss is also followed by a bone resorption alveolar[2].

In bone tissue engineering, there are several kinds of procedures, autograft, allograft, xenograft and alloplastic graft. Autograft is a standard procedure which is good for bone tissue engineering done,

however, the cost for this procedure is fairly expensive, there is a limited supply of tissue due to the high morbidity rate in donor specific area that can be used to donor[3]. Allograft is an embedded non-vital bone from cadaver and processed using a freeze-drying method in order to eliminate the water content in the bones. This method avoids tissue death, but can be potentially increased risk of transmission of disease and excessive immune response of recipient[4]. Xenograft is a method of bone grafts derived from other species than human[5]. Xenograft can help the process of bone regeneration because it does not require additional surgery, lower morbidity, and reduce the risk of disease transmission[1]. Alloplastic bone graft bone graft is a method that uses synthetic materials and are osteoconductive for osteogenesis process[6].

In recent years, innovations have been developed bone tissue engineering and as a result the focus on physicochemically biomaterial scaffold is a network engineering design for cell attachment, proliferation, differentiation, and tissue formation of certain organs[7]. Scaffold is a medium for the synthesis of the extracellular matrix (ECM) and become an attachment and growth of new cells. Thus, the scaffold is made of biodegradable materials that can be metabolized in the body and eventually disappear when the new cells can grow a lot, healthy and survive. Some polymer materials, which have been developed in tissue engineering is chitosan[8].

Chitosan is amino polysaccharides (poly-1,4-D-glucosamine), and are widely used as engineering polymers in tissues[9]. This polymer has been considered as a material that has many functional advantages[10]. Chitosan is used both in tissue engineering because of the biodegradable nature, biocompability, anti-bacterial, for wound healing and is bioadhesive[11], however chitosan membranes have very rigid and brittle which means it has a low mechanical resistance. So as to optimize the mechanical resistance force chitosan, need additional carbonate apatite as an additional polymer material as carbonate apatite has the same physicochemical properties as bone[12]. Carbonate apatite is the main mineral components of hard tissues of human bones and teeth. Then, carbonate apatite material is increasingly being used as a biocompatible material for medical purposes in bone tissue engineering. Carbonate apatite developed showed good biocompatibility on animal trial[13].

*Biomaterials* nanostructured significantly improve cellular function leading to increased osteoinductivity and osseointegrativity, because of osteogenic cells interact with minerals and proteins are nano[14]. In the form of nanoscale, the scaffold can increase the overall surface area, surface to volume ratio, and violence of surface[15], which can increase the adhesion between osteoblasts with covering all scaffold surface[16]. The more surface area can improve the absorption of proteins, cell adhesion and cell growth[17], therefore, more and more nano-materials with good biocompatibility synthesized and used for applications biomedically[13].

Bone healing was marked by a series of cellular and molecular processes. Network transformation consists of resorption and formation of hard tissue and soft tissue. In the regeneration of skeletal bone, osteoblasts play a role in bone formation and resorption of osteoclasts role in bone[18]. Growth factor usually

stored in the extracellular matrix (ECM) and in case of trauma, growth factor actively released by ECM, cells and platelets to do regeneration[19]. The growth factors are mitogens with important effects on cell function.

Growth factors such as fibroblast growth factor-2 (FGF-2) plays an important role in the process of bone regeneration.FGF-2 is a polypeptide that can be found in various tissues including bone tissue. FGF-2 is involved in many biological processes of embryonic development that regulate cell proliferation, migration and differentiation, homeostasis set up maintenance and repair tissues[13]. FGF-2s have a specific effect on osteoblast maturation, as well working to stimulate the differentiation of cells to withstand osteoprogenitor or osteoblast different maturation[20]. Osteoblast cell proliferation will be increased on the first day then, will decline back. Increased proliferation of osteoblasts will happen to the day-7[21].

Based on the explanation and understanding of the mechanisms of the relationship between the bone regeneration process with chitosan nano-carbonate apatite scaffold, it is very important to improve the methods for the next generation of scaffold that can indicate bone regeneration efficiency and in vitro. Based on this background, the authors wanted to examine the expression of fibroblast growth factor-2 (FGF-2) on osteoblast cells grown in chitosan nano-carbonate apatite scaffold.

## MATERIALS AND METHODS

The research is a laboratory experimental research design with post test only control group design. The samples are femur osteoblast cells from male rats, chitosan nano-carbonate apatite scaffold.

Planting 2 x 106 osteoblast cells on chitosan nano-carbonate apatite scaffold. Then the samples were incubated for in immunohistochemistry (IHC), the samples were washed with a solution of PBS pH 7.4, and then incubated with antibody monoklomal using anti-FGF-2's observations on FGF-2, for 60 minutes. After that, incubated back by using a HRP conjugated anti-rabbit for 40 menit.inkubator for 3, 5, and 7 days. After incubation, the samples were made paraffin blocks. Before the observations using a sample washed using dH2O, then performed the Counter staining using Meyer Hematoxilen incubated for 10 minutes and washed using tap water. Samples were dried by dianginkan. Then observed using a light microscope with a magnification of 1000x.

## RESULTS

This study uses osteoblast cell cultures were obtained from Biomedical Laboratory Faculty of Medicine, University of Brawijaya, Malang. Osteoblast cell cultures were then implanted in the nano chitosan - carbonate apatite scaffold with observation time on day 3, 5 and 7.

Incubation was performed in 7 samples for each group of observation time. From the observation and calculation of expression of FGF-2 data obtained as follows:

| samples | Total Expression | | |
|---|---|---|---|
| | Day 3 | Day 5 | Day 7 |
| 1 | 9 | 11 | 18 |
| 2 | 6 | 11 | 21 |
| 3 | 7 | 13 | 18 |
| 4 | 9 | 11 | 19 |
| 5 | 6 | 13 | 17 |
| 6 | 7 | 16 | 12 |
| 7 | 9 | 13 | 15 |
| **Average** | 7.5714 | 12.5714 | 17.1429 |

**Table 1.** The results of the expression of FGF-2 at the time of observation day 3, 5 and 7.

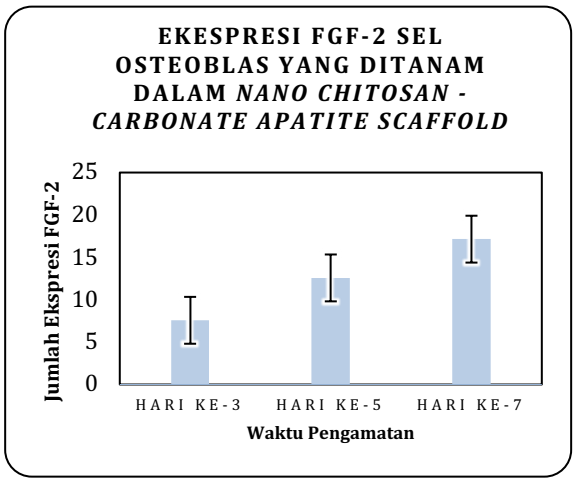


**Figure 1.** The observation of the expression of FGF-2 at the time of observation day 3, 5 and 7.

Observation time on day 3, 5 and 7 was conducted to evaluate the expression of FGF-2 on osteoblast cells grown in chitosan nano - carbonate apatite scaffold. The observation of the expression of FGF-2 on day 3 using immunohistochemistry (IHC) are shown in Figure 2.

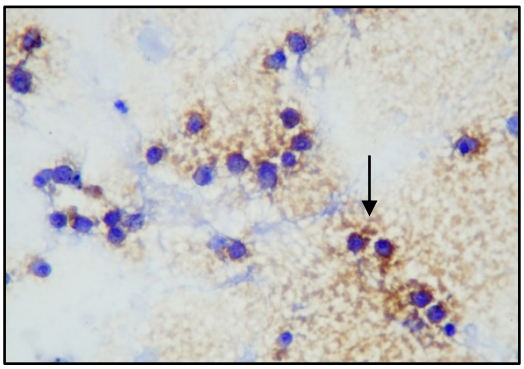

**Figure 2.** The observation of the expression of FGF-2 in cultured osteoblast cells grown in chitosan nano - carbonate apatite scaffold with an observation time of day 3.

The observation of the expression of FGF-2 on day 5 using immunohistochemistry (IHC) are shown in Figure 3.

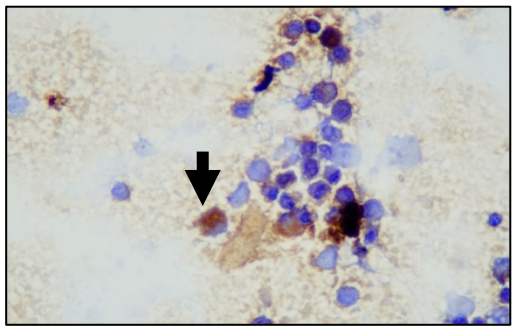

**Figure 3.**The observation of the expression of FGF-2 in cultured osteoblast cells grown in chitosan nano - carbonate apatite scaffold with an observation time of day 5.

The observation of the expression of FGF-2 on day 7 using immunohistochemistry (IHC) is shown in Figure 4.

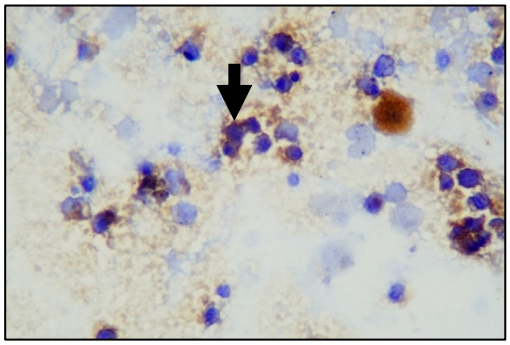

**Figure 4.** The observation of the expression of FGF-2 in cultured osteoblast cells grown in chitosan nano - carbonate apatite scaffold with an observation time of day 7.

## DISCUSSION

An evaluation similar to other types of scaffold in vitro on bone tissue engineering techniques typically begins with a chitosan-based microstructural characterization scaffold in terms of size, shape and interconnectivity pores[22]. In the form of nanoscale, the scaffold can increase the overall surface area, surface to volume ratio, and violence of surface[15], which can increase the adhesion between osteoblasts with covering scaffold surface[16]. The more surface area can improve the absorption of proteins, cell adhesion and growth[17].

Chitosan as bone tissue engineering materials can support the adhesion and proliferation of bone-forming osteoblasts and the formation of mineralized bone matrix in vitro. In vitro studies by Sheeny (2014), have shown that chitosan can improve the adhesion and proliferation of osteoblasts. Osteoblast cell culture that has been embedded in the scaffold with ECM biomaterials chitosan can form the mineralized to produce bone tissue.

Carbonate apatite is the main mineral components of hard tissues of human bones and teeth. This material is increasingly being used as a biocompatible material for medical purposes, because the carbonate apatite has physicochemical properties similar to bone. The content of carbonate apatite carbonate deposits may be optimal in apatite and apatite crystals can trigger a chemical bond between the biomaterials and bone tissue, which can affect the formation of bone[23]. Based on the explanation, the second mate and carbonate apatite chitosan is a good material for use as biomaterials engineering of bone tissue, so that the research conducted by embedding a culture of osteoblasts into chitosan nano-carbonate apatite scaffold.

Based on the results obtained, the acquisition increased expression of FGF-2 at the time of observation day 3, 5 and 7. The results of the observation on day 7 showed the highest increase of the expression of FGF-2. According to Tiffany et al., (2012) in the first hour until the 3rd day of the start of the process of cell proliferation characterized by the release of pro-inflammatory cytokines and growth factors such as interleukin (IL) -1, IL-6, TNF-α, FGF, PDGF and TGF-β1 from the systemic circulation and inflammatory cells that initiate signaling for matrix deposition and activation of progenitor cells. At the time of observation day 3 in the treatment group 1 obtained an average value of the expression of FGF-2 at 7.5714 which showed a proliferation of osteoblasts.

The expression of FGF-2 at the time of observation day 5 in the treatment group 2 gained an average value of the expression of FGF-2 amounted to 12.5714 which shows the increase osteoblast cell proliferation. According Planell et al., (2009) the proliferation of osteoblasts increased on the first day up to the 2nd and after that it will decline again, until the 7th day there will be an increase in proliferation. And based on the results obtained seen an increase higher than the expression of FGF-2 at the time of observation of the 7th day of the treatment group 3 obtained an average value of the expression of FGF-2 amounted to 17.1429.

Osteoblast cell culture is embedded in the carbonate apatite nano chitosan-containing chitosan scaffold that is biocompatible and provides an excellent space for osteoblast adhesion. Cell adhesion is very important to maintain the activity of cells and multicellular structures. Chitosan is non-toxic, antibacterial, and biocompatible. So that chitosan does not change the morphology or viability of osteoblasts, therefore physiologically degradable chitosan. Having used his scaffold to the osteoblast cells, the scaffold is degraded in some time. In the process enables the formation of ECM scaffold degradation due to degradation occurs simultaneously with the formation of new tissue. The role of macrophages in the inflammatory response is required in the degradation process ini24.

Osteoblasts play a role in the growth of bone cells (Barrett et al. 2010) and osteoblasts are very common with the fibroblast growth factor that is a whole of genes expressed in the fibroblast growth factor is also expressed in osteoblasts. Pre-osteoblasts are bone progenitor cells that are stimulated by growth factors such as Fibroblast Growth Factor (FGF-2) and migrate to a specific location to grow and differentiate into osteoblasts. The secretion of other growth factors including BMP, GDF, IGFs, TGF-β and osteoprogenitor occur at a later stage (5-21 days) and return to normal levels during remodelling25.

Based on the explanation, then when culture osteoblast cells embedded in nano chitosan-carbonate apatite scaffold, osteoblasts will experience accelerated growth by biomaterial chitosan and carbonate apatite, so that, when osteoblasts growth will be stimulated by multiple growth factors one of which is fibroblast growth factors-2 (FGF-2). Thus, the expression of FGF-2 will increase when osteoblasts were proliferating and growing.

## CONCLUSION

Based on these results it can be concluded that an increase in the expression of FGF-2 on osteoblast cells grown in chitosan nano-carbonate apatite scaffold on days 3, 5 and 7.

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