World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

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A potential approach for *in vitro* bone tissue engineering: expression of BMP-2 and FGF-2 on nano Ch-CA scaffolds

Maretaningtias Dwi Ariani *, Ruana Puteri Fizhillah, Nabila Izas Herninda, Harly Prabowo and Nadia kartikasari

Department of Prosthodontics, Faculty of Dental Medicine, Universitas Airlangga, Surabaya 60132, East Java, Indonesia.

World Journal of Advanced Research and Reviews, 2024, 21(01), 1046–1052

Publication history: Received on 05 December 2023; revised on 10 January 2024; accepted on 13 January 2024

Article DOI: https://doi.org/10.30574/wjarr.2024.21.1.0138

Abstract

Background: Alveolar bone resorption after tooth extraction is a physiological process that cannot be avoided. The traditional approach to treating bone defects is frequently ineffective, but gene therapy, which aims to maintain the local concentration of bone growth factors at a therapeutic level, is a promising method for expediting bone defect repair. One of the polymer materials, which have been developed in bone regeneration is chitosan (Ch). Ch in the form of nanoparticles is neutral, not toxic, and has constant stability. To Enhance the bone regeneration properties of the graft substitution, nano chitosan-carbonate apatite scaffold (nano Ch-CA scaffold) combined with bone morphogenetic protein-2 (BMP-2) and fibroblast growth factors-2 (FGF-2) were developed. BMP-2 and FGF-2 have osteoinducing properties and functions in regulating the process of proliferation, differentiation, and apoptosis of osteoblasts. Purpose: To observe the expression of BMP-2 and FGF-2 on a nano Ch-CA scaffold that promotes osteogenesis *in vitro*. Materials and Methods: Forty-two nano Ch-CA scaffolds were used and divided into 2 groups, namely group I containing osteoblast cells seeded on nano Ch-CA scaffold with BMP-2, and group II containing osteoblast cells seeded on nano Ch-CA scaffold with FGF-2. Expression of BMP-2 and FGF-2 were examined on days 3, 5, and 7. Each time of examination consisted of 7 nano Ch-CA scaffold. An immunohistochemistry (IHC) test was used to assess the levels of BMP-2 and FGF2 expression on nano Ch-CA scaffold *in vitro*. Results: The cylindrical nano Ch-CA scaffold that was prepared had a uniform size distribution. The BMP-2 and FGF-2 expression levels were significantly increased on days 3, 5, and 7 of observation. It is possible to infer that encouraging osteogenesis on nano Ch-CA scaffold *in vitro*. Conclusion: The nano Ch-CA scaffold shows good handling properties when combined with BMP-2 and FGF-2. The osteogenic capabilities of osteoblast cells are effectively promoted *in vitro* by the expression of BMP-2 and FGF-2 on a nano Ch-CA scaffold.

Keywords: Bone morphogenetic protein-2 (BMP-2); Bone tissue engineering; Fibroblast growth factors-2 (FGF-2); Nano chitosan-carbonate apatite scaffold (nano Ch-CA scaffold); Osteoblasts cell

1. Introduction

As a mineralized structure with a complex metabolism, bone tissue requires a structure that is tailored to the mechanical needs of the bone.[1] A live tissue that can encourage bone healing that is transplanted into a bony defect, either alone or in conjunction with other materials, is referred to as a bone graft.[2,3] Due to improvements in dental implantology and the increasing demand for the correction of cranial bony abnormalities, the use of bone grafts and substitutes has significantly expanded in dentistry in recent years. These bone or skeletal deformities may result from trauma, periodontal disease, surgical excision, cranioplasty, infection, congenital anomalies, or oral cancer.[2] The development of allografts, synthetic bone grafts, and new surgical procedures may have had an impact on the usage of bone transplants in recent years. Autogenous, allogeneic, and artificial bone grafts are popular types and sources of grafts.[2,4]

* Corresponding author: Maretaningtias Dwi Ariani

A bone substitute is a natural or artificial substance that serves the same function but typically only contains a mineralized bone matrix and no living cells.[4] None of the materials now available on the market have all the qualities that make up an ideal bone substitute material, such as minimal patient morbidity, simplicity of use, low immunogenicity, affordability, and angiogenic potential.[5-7] The drawbacks of autografts include the scarcity of graft tissue, associated pain, morbidity at the donor site, and the requirement for two surgical procedures, whereas the drawbacks of allografts include immune system rejection of the donor tissue by the recipient and worries about the transmission of diseases like HIV and hepatitis.[8,9]

Despite a lack of evidence-based research on indications and safety, there has been a growing push in the market to adopt innovative bone grafting materials, such as bone substitute products.[5] Thus, these issues warrant more study into the creation of novel materials utilized in bone grafting operations, which is strongly suggested by the continuous notable increases in demand for bone graft materials and the aging world population.[8,10,11] *A synthetic graft called scaffold serves as a filler for damaged areas and enhances bone regrowth. Cells and growth factors are frequently mixed with a scaffold to facilitate osteoinductivity and the process of tissue regeneration*.[12]

Artificial synthetic bone substitute materials are developed to closely resemble the biological characteristics of genuine bone to overcome potential immunogenicity and morbidity at donor sites. However, the only osteoconductive and osteointegration capabilities of currently accessible synthetic materials are seen. A naturally occurring polymer with biocompatibility and biodegradability is chitosan.

Nanoparticles made of Ch are neutral, non-toxic, and consistently stable. In cell culture, Ch encourages the development and differentiation of osteoblast cells and possesses advantageous properties like bacteriostatic, hemostatic, and cholesterol-lowering effects.[13] The bio-ceramic substance carbonate apatite (CA) has been developed and researched to serve as the preferred material for the building of artificial bone. The ideal material for bone repair surgery is CA because of its remarkable biosorption capabilities. With the addition of CA to Ch, the material's surface area for cell attachment rises and the combination of the two promotes rapid cell multiplication.[10] Because materials at the nanoscale are known to have enhanced cell functioning, we used a nano Ch-CA scaffold in this experiment.

Bone morphogenetic proteins-2 (BMP-2) and fibroblast growth factor-2 (FGF-2) are two growth factors that have an impact on this process. The BMP-2 protein family supports the growth of bone and cartilage while also having an impact on a variety of non-osteogenic processes. BMP-2 proteins have an osteogenic function, which makes them useful in many medical specialties, including dentistry.[1,14] Known for their capacity to promote the production of cartilage and bones, BMP-2 is a category of growth factors and cytokines. BMPs, which are a member of the transforming growth factor beta (TGF-ß) superfamily, have osteoinductivity qualities. Particularly, BMP-2 demonstrated highly potent osteoinductivity action. BMP-2 evolved into an osteoinductivity growth factor that is applied clinically in bone regeneration. Due to its pleiotropy, BMP-2 has been investigated to enhance fracture healing. BMP-2 stimulates angiogenesis, multiplies stem cells in the bone fracture, and induces osteoblast development.[15-17]

In vivo, bone development and regeneration are regulated by several growth factors. Growth factors, FGF-2, are essential to the process of bone regeneration. Numerous tissues, including bone tissue, contain a polypeptide known as FGF-2. The differentiation of cells to survive osteoprogenitor or unique osteoblast maturation is directly impacted by FGF-2, which also serves to increase cell survival.[18] FGF-2, a growth factor that promotes osteogenesis and neovascularization, is one of the agents used to regenerate tissue. It has been suggested that FGF-2 can promote bone growth. Additionally, several studies have shown that FGF-2 greatly encourages the production of new alveolar bone in people with chronic periodontitis when they participate in randomized clinical trials. It has also been shown in numerous studies that local application of recombinant FGF-2 combined with an adequate carrier induces bone growth at a bone defect site.[19] The goal of the current work is to observe how osteoblast cells placed in nano Ch-CA scaffold for alveolar bone regeneration exhibit increased BMP-2 and FGF-2 expression.

2. Material and methods

This study used laboratory research with a posttest-only control group design. The samples consisted of sixty-three nano Ch-CA scaffolds measuring 5 mm in diameter by 2 mm in height, along with osteoblast cells isolated from male rat femurs (The Biomedical Laboratory of the Faculty of Medicine, University of Brawijaya, Malang). Forty-two nano Ch-CA scaffolds were used and divided into two groups: group I contained osteoblast cells seeded on a nano Ch-CA scaffold with BMP-2, and group II contained osteoblast cells seeded on a nano Ch-CA scaffold with FGF-2. On days 3, 5, and 7, BMP-2 and FGF-2 expression levels were analyzed. Each examination period included seven nano Ch-CA scaffolds.

Each nano Ch-CA scaffold has been seeded with $2x10^6$ osteoblast cells. The samples were then cultured for 3, 5, and 7 days in an incubator before paraffin blocks were created. The sample was cleaned with PBS solution pH 7.4 before being examined using immunohistochemistry (IHC). It was then treated for 60 minutes with anti-BMP-2 and anti-FGF-2 monoclonal antibodies. After that, an additional 40 minutes of incubation with anti-rabbit HRP conjugated was performed. The samples were additionally rinsed with dH_2O, counter-stained with Meyer Hematoxylin, incubated for 10 minutes, and then washed with tap water. By aerating, the sample was dried. Afterward, it was examined using a 1000x light microscope.

3. Results

The following average values of BMP-2 and FGF-2 expression in osteoblast cells seeded on a nano Ch-CA scaffold were determined based on observations on days 3, 5, and 7 (Figures 1-3).

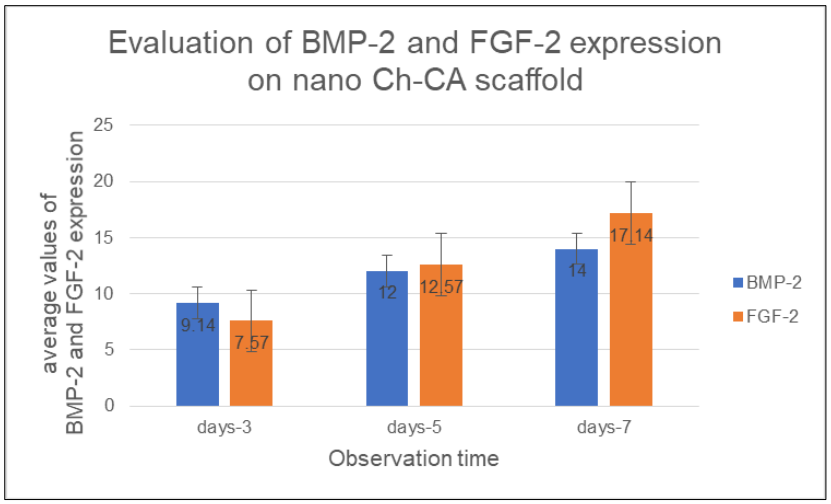

Figure 1 Observation of BMP-2 and FGF-2 expression of osteoblast cells seeded in nano Ch-CA scaffold on days 3, 5, and 7

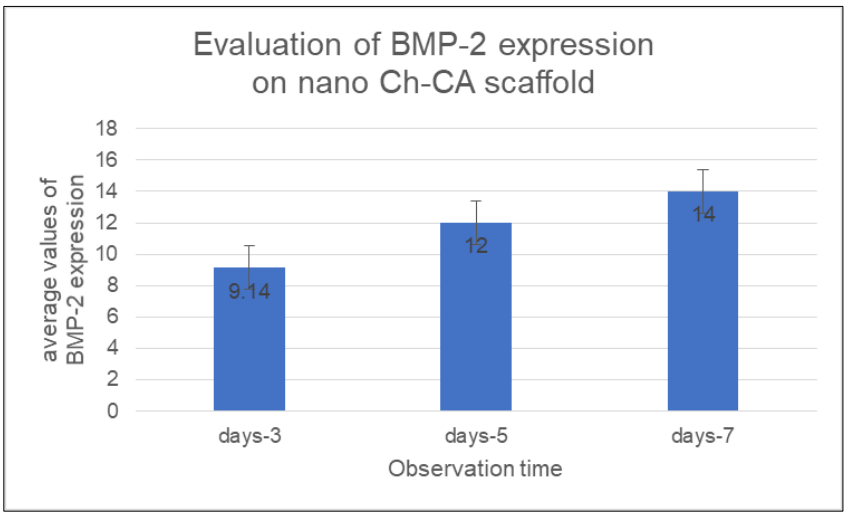

Figure 2 Observation of BMP-2 expression of osteoblast cells seeded in nano Ch-CA scaffold on days 3, 5, and 7

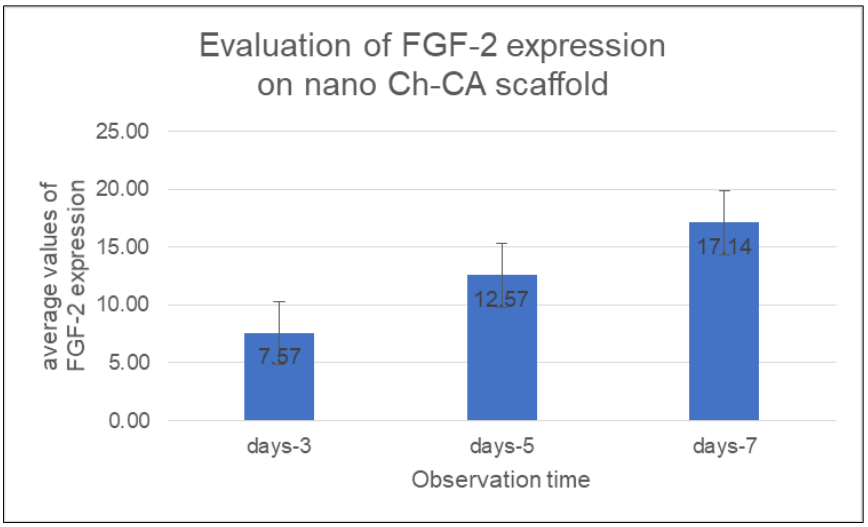

Figure 3 Observation of FGF-2 expression of osteoblast cells seeded in nano Ch-CA scaffold on days 3, 5, and 7

Figures 4-5 illustrate how immunohistochemistry (IHC) and 1000x magnification light microscopy were used to observe the expression of BMP-2 and FGF-2 on days 3, 5, and 7. The expression of BMP-2 and FGF-2, which are shown to be increasing, can be noticed in the microscopic appearance with visible IHC-stained osteoblast that appears as blue, round cells.

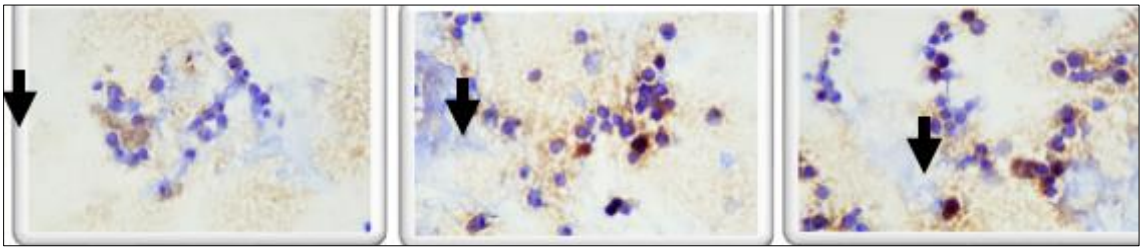

Figure 4 Immunohistochemical (IHC) picture of BMP-2 expression on nano Ch-CA scaffold on the 3rd, 5th, and 7th day of observation

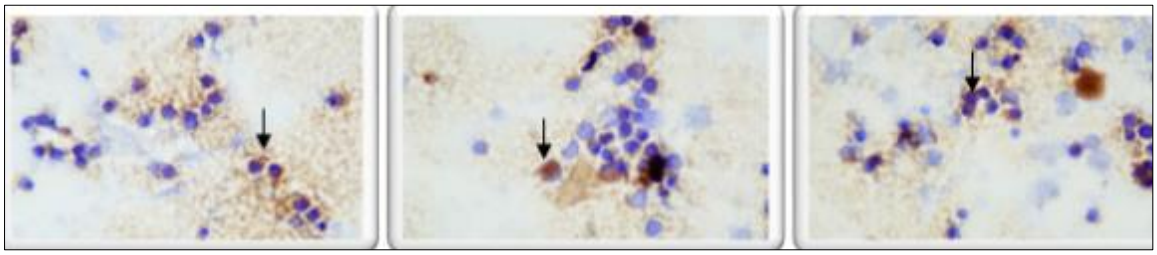

Figure 5 Immunohistochemical (IHC) picture of FGF-2 expression on nano Ch-CA scaffold on the 3rd, 5th, and 7th day of observation

4. Discussion

In cell culture, chitosan encourages osteoblast growth and differentiation and has beneficial properties like bacteriostatic, hemostatic, and cholesterol-lowering effects. 20,21 Comparing carbonate apatite to hydroxyapatite reveals advantages. Low-temperature processed carbonate apatite contains few bone crystals, which are thought to be resorbed by the body during metabolic processes. 20 It also possesses a high amount of carbonate apatite osteoconductive, which is predicted to promote the development of new bone.

Nano Ch-CA scaffolds feature an interconnected three-dimensional pore structure; neither form is brittle and can sustain osteoblast development and proliferation. 20,21 The osseointegration process is significantly influenced by the size and form of the pores. However, in this study, the nano Ch-CA scaffolds for nanoscale materials exhibit better functioning and simpler resorption. The addition of carbonate apatite to the nano chitosan scaffold increases the surface area for cell attachment osteoblast because the scaffold with high mechanical strength is more effective in supporting the proliferation of osteogenic cells.21

BMP-2 expression initially increased; it is thought to help with the differentiation of precursor cells into chondrogenic or osteogenic tissues. The multipotent mesenchymal progenitor cell lines are differentiated into osteogenic lineages by the prototypical subgroup of BMPs, BMP-2. 15,16

The objective of the current investigation is to determine whether osteoblast cells cultivated on nano Ch-CA scaffolds for alveolar bone regeneration express more BMP-2 and FGF-2 than other cell types.22 Following a lengthy process of adhering osteoblast cells to the nano Ch-CA scaffold's surface, the cells spread out across the material's surface. For anchorage-dependent cells to survive in the matrix, cell adherence is a fundamental prerequisite. It is the initial stage of a string of cell activities that also include cell migration, diffusion, proliferation, and differentiation. Extracellular matrix (ECM), which surrounds cells in vivo and has physical, biochemical, and micromorphological characteristics, may influence and govern the behavior and function of cells, leading to cell reactions. Additionally serving as the foundation for cell-cell communication with the outside world, cell adhesion is crucial for the growth of tissues.23

Male mouse femur primary cells are ingested into osteoblast cell culture more quickly than female mouse femur primary cells. Any imbalance disrupts bone remodeling, which is tightly controlled by a dynamic interaction of locally and systemically affecting elements. The parathyroid hormone (PTH), calcitriol, sex hormones, glucocorticoids, and thyroid hormones are examples of systemic variables that have an impact on remodeling. On the other hand, local factors consist of cytokines, prostaglandins, tumor growth factor beta (TGF-ß), and specific morphogenetic proteins. Nutrition, physical inactivity, chronic illness, and pharmaceutical medications are other factors that influence bone mass and quality.15,24

The cell culture was split into nine plates, each containing 2 ml of media, at the time the sample was taken. Following that, the samples were cultured for 3, 5, and 7 days in an incubator. Using nanoparticles as a benefit can deliver drug compounds well up to the smallest units of the body, and improve the efficiency of distribution, as well as the medication on the target, thereby increasing the therapeutic effect and reducing toxicity.

Immunohistochemical analysis demonstrated that BMP-2 levels increased in tissue that was available on days 3, 5, and 7 during the growth phase related to cell proliferation. As a result of the carbonate apatite being added to the chitosan scaffold, which increases the surface available for cell attachment, the research findings indicate that BMP-2 levels rise, promoting osteoblast growth.1, 22

In this study, the average level of BMP-2 expression was increased on days-3 to days-7 examination. Because BMP-2 has a significant impact on osteoblast cell differentiation *in vitro* and in vivo during bone formation, there is a faster increase on days 3 to 5 than on days 5 to 7. The synthesis of ALP and osteocalcin expression, which serve as markers of osteoblast differentiation, were used to categorize the ability of BMP-2 to cause differentiation from the beginning to the conclusion of marker differentiation.25

To enhance osteogenesis, many BMP-2 are produced from mineral-containing substrate osteoconductive bone. The study demonstrated a rise in ALP when BMP-2 was combined with a nano Ch-CA scaffold. This is relevant to recent studies that greatly enhanced BMP-2 levels when combined with nano Ch-CA scaffold. Observation days 3, 5, and 7 saw an increase in FGF-2 expression as a result of the acquisition, according to the results. The observation on day 7 revealed the greatest rise in FGF-2 expression. From the first hour to the third day after the start of the cell proliferation process, pro-inflammatory cytokines and growth factors like interleukin (IL)-1, IL-6, TNF-, FGF, PDGF, and TGF-1 are released from the systemic circulation, and inflammatory cells that start signaling for matrix deposition and activation of progenitor cells. At the time of observation, day 3 obtained an average value of the expression of FGF-2 at 7.57. During observation day 5, the expression of FGF-2 increased by an average value of 12.57, indicating an increase in osteoblast cell proliferation. From the first day until the second, osteoblast proliferation rose; following that, it started to fall. Proliferation grew up till the seventh day. Based on the outcomes, we observed a rise in the expression of FGF-2 at the time of observation on 7 days of observation. The expression of FGF-2 was averaged out to be 17.14.

5. Conclusion

These findings suggest that in combination with BMP-2 and FGF-2, the nano Ch-CA scaffold has good handling characteristics. By expressing BMP-2 and FGF-2 on a nano Ch-CA scaffold, osteoblast cells' ability to produce bone is successfully encouraged *in vitro*.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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