Potential syringetin-based nHAC/PLGA as bionano bone graft to improved osteoinductivity and osteoconductivity

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ABSTRACT

Background: According to the data of Riskesdas in 2013, fracture incident rate in Sulawesi gets a 16% increase. The most common fracture incident happens to elderly age group, and its predominant cause is a traumatic incident like falls and traffic accident. Treatment now such as ORIF and External Fixation is far from cheap and has a high risk to the patient. Syringetin-based nHAC/PLGA usage can be a new bionano bone graft because it can target specific cells to increase osteoinductivity in bones.

Discussion: Syringetin-based nHAC usage has some targets like BMSC. Syringetin-based nHAC affects in increasing the expression of BMP-2 and miRNA-21. BMP-2 and miRNA-21 are factors that take part in osteoblast proliferation and differentiation. That expression increase is marked by ALP activity and osteocalcin increase.

Conclusion: Significantly, the usage of syringetin-based nHAC/PLGA can increase the proliferation and differentiation rate of BMSC to osteoblast.

Keywords: Syringetin, nHAC/PLGA, Osteogenesis, Osteoinductivity

INTRODUCTION

Fractures are defined as discontinuity of bone structure and are generally a common occurrence in the elderly age group; the incidence of fractures in the elderly tends to increase with age. The prevalence of fractures is 4.4% of the population of South Sulawesi and 5.8% of in Indonesian population. Increased prevalence of fractures in South Sulawesi was also reported in Riskesdas Indonesia 2013 by comparing Riskesdas in 2007 and 2013; there was a 16% increase in the incidence of fractures from 3.7% to 4.3%. The incidence of fractures in South Sulawesi often occurs in the age group 65-74 years (11.1%). In South Sulawesi, the incidence of fractures in Indonesia is lower in the young group than the older one.

Gender also influences on fracture rates, studies of hip fracture incidents in Japan and Singapore indicate that the incidence of hip fracture is 2.6 to 3.5 times more common in women. There have also been reported increases in Japan over the past 30 years. The increment was concluded due to the increasing population of the elderly in Japan. Increasing the elderly population occur in Indonesia, and the prevention and treatment of fractures need to be improved to maintain and improve the quality of life of the elderly in the future. Data taken from Olmsted County reports the most frequenting fractures are vertebral fractures with an age range of 85 and above in both genders. Other than vertebral fracture (24%), leg fracture (10.5%) is the second most common fracture and the third most common is rib fracture (10.3%).

The cause of the fracture in the report from Amin et al. divided into four, namely severe trauma (33.4%), moderate trauma (60.2%), pathological (2.1%), and those not included in that category (4.3%). From this study, severe trauma usually causes leg fractures (20%); moderate trauma and pathological conditions often cause thoracic vertebral fractures (32% and 38% respectively).

Treatment of fracture patients can be classified as non-operative and operative handlers. Handling of non-operative fractures can be done conservatively such as elevation, the application using ice, and splints. Management of orthopaedic operations such as open reduction and internal fixation (ORIF) and external fixation. Treatment after operating and non-operating fractures are currently only focusing on the reduction of short-term complications and long-term. Both postoperative treatment and non-operation strategies aim to relieve both the pain and venous thromboembolic state. However, there is still no bone graft treatment that has the target of increasing osteoinductivity in the fractured bone.

Inducing the osteoblast will improve the process of forming osteoblast cells.
build new bone tissue so that it will reshape the bone tissue structure to become new. Various limitations of the treatment of fractures offer opportunities for the development of new therapies. One of the natural ingredients that have the effect of increasing osteoinductivity is syringetin. Syringetin is a flavonoid derivative that has a mechanism of action on improving BMP-2 and miRNA-21 to accelerate the process of differentiation and maturation of osteoblast. The low bioavailability of syringetin was enhanced by the use of encapsulation of HAC/PLGA nanoparticles. Those nHAC / PLGA also has excellent properties as a bone graft for fractures. With these advantages, syringetin-based nHAC/PLGA has the potential to be a bio-nano bone graft in the treatment of fractures.

**METHOD**

The study was a literature review that consisted of relevant journals from search engines such as Pubmed, Google Scholar, and Proquest. Writers searched the keywords "Syringetin", "Nanohydroxyapatite", "PLGA", "Bone remodelling", "Fracture", "Osteogenesis", and "Osteoinductivity". The inclusion criteria were all syringetin, nanohydroxyapatite, PLGA, osteogenesis, osteoinductivity. The collected references then noted and analyzed for validity and reliability, interpreted and compiled into a scientific literature review.

**DISCUSSION**

**Syringetin: Flavonoids Induces Osteogenesis**

Flavonoids are compounds that can be extracted from various types of fruits and vegetables. Some flavonoids have been shown to affect osteoblastic differentiation, such as daidzein, genistein, quercetin, kaempferol, naringin, myricetin, and diosmetin. Syringetin (3,5,7,4'- tetrahydroxy-3',5' dimethoxyylavone) is a derivative of flavonoids, which can be found in grapes and khamar. The concentration of syringetin found in grapes reached 3.22%.8

Hsu et al. research showed that syringetin could increase BMP-2 synthesis and activate SMAD1/5/8 and ERK1/2. These effects can contribute to its activity to induce maturation and differentiation of osteoblasts followed by an increase in bone mass.9

**Nanohydroxyapatite and Its Application**

Nanohydroxyapatite collagen (nHAC) is a nanoparticle delivering drugs for bone therapy. It is frequently applied because of its biocompatibility, bioactivity, and osteoconductive properties.10 Various studies have shown that it can be absorbed by osteoblasts and affect the fusion of osteoclast mononuclear precursors. These properties make nHAC potentially able to deliver drugs directly to the desired tissue and are useful as a tool to improve the treatment of several bone diseases such as osteomyelitis and cancer.11

One of the essential ingredients of hydroxyapatite derived from natural materials is an eggshell. Hydroxyapatite (EHA) of eggshell formulation showed excellent biocompatibility and material properties in vitro. It is a promising graft material with unique features for grafting. It is hydrophillic, absorbs fluid and blood around it, making it easy to handle and put on the surgical site. It can be implemented as a graft material for grafting bone defect secondary to periodontal disease, trauma, tooth extraction, intra-bone defects, sinus removal procedures, and so on.12

Poly lactic-co-glycolic acid (PLGA) is a copolymer compound which is synthesized through the opening of the copolymerization ring of two different monomer compounds that form cyclic dimers (1,4-dioxide-2,5-diones) of lactic acid and glycolic acid. The ratio of the amount between lactic acid and glycolycalryc is 50:50. PLGA has excellent biocompatibility and biodegradability properties. It is useful in making nanoparticles.13,14

In reconstructive or regenerative bone surgery, ideal material functions not only as a carrier for drug delivery but also as a porous scaffold for cellular activity. So that the combination of nHAC with type one collagen and PLGA will be a useful scaffold for bone cell growth, and drug delivery such as syringetin.15

**Fracture healing mechanism and Bone Remodeling**

Fracture Healing divided into several steps. First, there is a hematoma and inflammation at the site of the fracture; second, chondrogenesis begins and callus formation occurs (cells that become vascular bone was also at this stage); third, osteoblast cells begin to form hard bones and connect the two broken bone segments (osteogenesis); fourth, bone remodelling occurs with the ended result of a stronger lamellar bone structure.16-18 The four steps above can also be divided into two phases, namely the anabolic phase (the cell recruitment phase and matrix mineralization) and the catabolic phase (the accumulation phase of mineral accumulation and vascularisation).18

Bone remodelling belongs to the catabolic phase. In that phase, osteoclasts begin to absorb the
matrix which has been mineralized during the osteogenesis phase. In this phase too, osteoblasts and osteoclasts work side by side (coupled remodelling); structure and vascularisation marrow hematopoietic tissue remodelling extensive also occur in this phase (Figure 1).

A critical compound in the fracture healing and bone remodelling is Bone Morphogenic Protein 2 (BMP-2). Mouse with the lack of expression of BMP-2 reported having a defect in differentiation and the proliferation of osteoblasts. Lack of BMP-2 expression has also been reported to cause osteoblasts not activated at fracture healing sites. Based on the above data, it can be concluded that lack of BMP-2 expression causes disruption of osteoblast production and function, and as a result, interferes with the process of osteogenesis (callus not fully formed) and bone remodelling is inhibited.

Nanohydroxapatite Construction and Administration Mechanism

Nanohydroxyapatite (nHA) will be used as a scaffold for the syringetin modality. Some reasons for selecting nHA have been discussed earlier in this article. There is also research by Wang et al. states that nHA can distribute evenly and in a uniform form. In the first 24 hours, modalities constructed with nHA, as much as 55% and the remainder were released slowly over a period of three days (the modality used by Wang et al. Was insulin); it states the ability of nHA in the equitable distribution of modalities, and the ability of sustained release is good considering fracture healing usually lasts long enough.

Construction begins by preparing the syringetin pattern in suspension, then proceed with the insertion of the nHA (scaffold) into the suspension modality with a vacuum suction of 0.1 MPa for 10 minutes, and mixed with a vortex mixer for 10 minutes. Syringetin in mixed suspension which is not bound to nHA can be separated after centrifugation of the suspension. Many syringetins that bind to nHA can be remodelling by subtracting the initial total syringetin with the rest of the syringetin.

As discussed in the introduction, no bone grafting treatment increases the osteoinductivity of the fractured bone. Administration of the syringetin modality will be carried out in conjunction with bone grafts inserted during ORIF (open reduction and internal fixation) orthopaedic surgery. Bone graft with this syringetin into the category of bone graft biosynthetic.

Mechanism of Action of Syringetin-Based nHAC/PLGA

Syringetin Effect on Osteoinductivity

Syringetin has osteoinductivity property in inducing differentiation of pre-osteoblast into late-osteoblast. An induction increase in pre-osteoblast differentiation will increase new bone tissue formation. Osteoinductivity property of syringetin comes from the induction of BMP-2 and Runx2 expression. Neither the specific target nor the mechanism of BMP-2 induction by syringetin is known until now. Ya-Ling Shu et al. stated that administration of syringetin to human fetal osteoblastic (hFOB) cell and MC3T3-E1 increase their BMP-2 expression. Basic helix-loops-helix (bHLH) protein and E-box protein binding will activate BMP-2 expression. Both bHLH protein and E-box protein specifically regulates BMP-2 gene expression on mesenchymal stem cell. Expressed BMP-2 then will bind to BMP-2 receptor and form heterodimer complex. Heterodimer complex activates two types of signal transduction that are Smad-independent and Smad-dependent. In Smad-independent signal transduction, there is p38 activation by mitogen-activated protein kinase (MAPK) that enables Runx2 to control mesenchymal stem cell differentiation and TAK-1 (TGF-β Activated Kinase-1) to regulate bone formation.

In Smad-independent signal transduction, TGF-β inducted receptor binds TRAF6 and ubiquitin to the cytoplasm to activate TAK-1 (TGF-β Activated Kinase-1) and MAPK (Mitogen-Activated Protein Kinase). TAK-1 and MAPK activation play a role in regulating mesenchymal stem cell differentiation and bone formation. Activated TAK-1 and MAPK trigger JNK and p38

Figure 1. Bone remodeling
phosphorylation. p38 and JNK will translocate into nucleus plasma and there they will form a complex with these transcription factors (Ras-Raf-MEK-Erk, MAPK JNK-c-Jun N terminal kinase, activating transcription factor-2 (ATF-2), p38 MAPK, SH2 domain-containing sequence A (SHCA), and extracellular signal-regulated kinase (Erk)); these complexes will then increase gene expression. 

Aside from Smad-independent activation path, heterotetramer complex can activate Smad path through R-Smad phosphorylation (Smad 1, 5, and 8). Smad 1, 5, and 8 can induce other TGF-β transducing protein signals. R-Smad activation leads to heteromeric complexes forming with C-Smad and their translocation into the nucleus. Those heteromeric complexes will bind with some other transcription factors [Runx2/Cbfα1 (core-binding factor alpha 1), Otx (Osterix), Dlx5, and Msx2 (msh homeobox homolog 2)]. Lastly, those molecules play a role in osteogenesis induction (Figure 2). The increment of BMP-2 expression marks syringetin effect. Besides, there are increase in alkaline phosphate (ALP) activity, an increase in osteocalcin expression, and type-1 collagen synthesis. 

Ya-Ling Shu et al. research showed that there is an increase of BMP-2 expression on MC3T3-E1 (osteoblast precursor cell line from Mus musculus) mice and hFOB cell (osteoblast cell line from human fetus cell). Increase in syringetin regulation can be observed 3 hours after injection of 20µM, and its maximal expression is noted after 12 hours. After 12 hours, the rise of BMP-2 expression follows a time-dependent and dose-dependent manner. Syringetin inducts BMP-2 dependent pathway (Figure 3). 

Increase in osteoblast proliferation can be tested by observing ALP and osteocalcin activity. ALP is a biological marker secreted in the early phase of osteoblast proliferation. Osteocalcin is the other biological marker that plays a role in evaluating osteoblast proliferation in the termination phase. When syringetin is injected into MC3T3-E1 mice and hFOB cell, ALP and osteocalcin increase is expected. ALP activity increases after 20µM syringetin administration with the dose-dependent manner in 48 hours and 72 hours. ALP increase marked the stimulation of pre-osteoblast proliferation into early osteoblast. After that, early osteoblast will develop into late osteoblast and then osteoblast itself (Figure 4).

The development phase of the late osteoblast is marked biologically by osteocalcin. Syringetin administration on MC3T3-E1 mice and hFOB cell can increase late osteoblast to osteoblast cell proliferation rate. This increase is marked by the rise in osteocalcin secretion after 48 hours and
research compared syringetin administration with noggin administration as a negative control that will be tested with immunoblotting analysis. The result showed that SMAD1/5/8 and Erk1/2 was much thicker on syringetin administration than that of noggin (BMPR-2 inhibitor) (Figure 5).

**Nanohydroxyapatite effect on increased osteoinductivity**

An important characteristic that nanohydroxyapatite have is osteoconductivity. Osteoconductivity helps in new bone tissue formation on a surface in an ongoing fracture healing. Nanohydroxyapatite has hydrophilic nature and helps osteogenic cell be more adhesive, proliferative, and differentiative on forming bone tissue. Osteogenic cells are much more prone to apoptosis in hydrophobic surface than that of a hydrophilic.

Tong et al. study used human osteoblast-like cell (SaOS-2) that was cultured into carbonated nHA (CHA) on polyhydroxybutyrate-co-hydroxy valerate (PHBV). Proliferation, morphology, and ALP were evaluated in 14 days. After 14 days, the result showed that ALP was found significantly increased in CHA/PHBV than in PHBV. Although both CHA/PHBV and PHBV shows increased adhesiveness, proliferation, and cell distribution. This reflects the use of CHA/PHBV increase ALP expression in cells. Zhang et al. study are about nHA/PCL activity with weight ratio difference on nHA and PCL to human fetal osteoblast (hFOB). The result showed that there was ALP activity and mineral matrix synthesis increase compared to control.

**PLGA Role in Half Time Enhancement**

nHAC/PLGA use for encapsulation could increase the half time of syringetin. Xing Wang study used a compound which was the same characteristic as syringetin and showed the use of nHAC/PLGA increase its half time. nHAC method for encapsulation showed that there was a 93% compound release in 24 hours. While in nHAC/PLGA showed 46% compound release in 24 hours and constant release for 5 days (Figure 6).

**Citocompatibility dan bioactivities of nHAC/PLGA**

The effect of nHAC/PLGA can affect the differentiation process of BMSC. Arumugam et al. research the differentiation and maturation degree of MSC using ALP and osteocalcin (OCN) as biological markers. The result shows an increase in ALP on day three until day fourteen after nHAC/PLGA administration; there is no difference in
ALP activity after day fourteen if compared with nHAC administration. Osteocalcin shows no significant differences in day three, day seven, and day fourteen.23

**Syringetin-based nHAC/PLGA Advantages**

Osteoblast can be inducted by some medication to cure bone disorder24 (e.g. osteoporosis), but those medications have potential side effects to the body due to toxicity.25 Polyphenol derived from plants can minimalize that toxicity for bone healthiness.26

According to some research, syringetin showed anti-diabetic activity and was used to cure diabetes in China and India traditional practices.27 Syringetin toxicity was researched with the calorimeter method using MTT reagent based on formazan blue detection; the result showed the non-toxic effect on mMSC. Even though many polyphenols are reported having antidiabetic, antioxidant, and anticancer activity, some has been tested for its osteogenic activity. Syringetin can induce osteoblast differentiation on the level of cell; it is evidenced by increased ALP activity and increased the accumulation of calcium deposit in mMSC.21

**CONCLUSION**

Syringetin-based nHAC/PLGA as a bone graft could increase the rate of proliferation and differentiation of BMSC into osteoblast cells in the treatment of fractures. Significantly, it is able to increase cell proliferation by increasing BMP-2 and miRNA-21 expression. Increased expression of BMP-2 is marked by increased activity of ALP and an increase in OCN expression. Increased expression of miRNA-21 will inhibit the expression of Smad7 which plays a role in inhibiting the activation of Runx2.

Its usage as encapsulation one may increase the half-life of syringetin and increase the rate of proliferation and differentiation of cells stem cells marked by an increase in ALP and OCN. The existence of an increase in the rate of proliferation and the length of half-life can be a treatment for fractures in the elderly because in the elderly, there is a decrease in the rate of bone remodelling. So expect nHAC/PLGA-based syringetin can be innovation bionano bone graft to pen n Subscribe fractures in the elderly.

**DISCLOSURES**

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