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REVIEW ARTICLE

Chitosan as Bone Scaffold and Graft Materials for Bone Regeneration: A Systematic Review

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Abstract Chitosan is a natural biodegradable polymer made from crustacean exoskeletons (shrimp and crab). Recently, natural material is preferably used in order to prevent any side effects from synthetic material. Previous research showed that chitosan has anti-bacterial properties, which can act as a bone graft scaffold material to increase in bone regeneration process. This article discusses the antibacterial effect of chitosan in the bone regeneration process. Bone graft consists of many primary graft materials which focus on alloplastic graft composite type. Bone graft is related to bone regeneration which associated with the process of secondary/indirect bone healing. The main trait of bone graft material is that it is not toxic to the chitosan's host cell. Chitosan's anti-bacterial effects can be associated with one of the three phases of bone defect healing, which is inflammation phase that acts to prevent bacterial invasion as an infectious disease in bone injury. Because of this, it can accelerate the polarization of macrophage M1 (secrete pro-inflammatory cytokine) to macrophage M2 (secrete anti-inflammatory cytokine) that relates to osteoblastogenesis. Osteoblastogenesis relates to the increase of osteoblast synthesis, deposition and mineralization of extracellular matrix leading to the maturation of osteoblast become osteocyte called ossification process.

Keywords: Chitosan, anti-bacterial, bone graft, bone regeneration, Infectious disease.

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Introduction

Chitosan is a natural biodegradable polymer which made from crustacean exoskeletons (shrimp, crab, lobster, and insect). Chitosan itself has an important position as a biomaterial because of its abundance, versatility, and unique properties (biodegradability, biocompatibility, non-toxic, hydrophilic, anti-bacteria and anti-fungal properties, and it also has an effect on wound healing process). With the presence of β-1,4 glycosidic bond with D-glucosamine and N-acetyl-D-glucosamine make chitosan easier to be modified by chemical reaction with high elasticity level, high flexibility, and low respond of inflammation. The process to obtain chitosan is by partial deacetylation of chitin using chemical or biological method. Degree Deacetylation (DD) determines the molecular weight of chitosan. The lower the deacetylation process, the higher chitosan molecular weight, but with the decrement of chitosan solubility against

traditional solvents. Chitosan is degraded into amino sugar products that are not harmful to the body. In fact, metabolites product will get absorbed completely by the human body. Research proved that chitosan is a biomaterial fabrication for tissue engineering, bone tissue engineering, intervertebral disc tissue engineering, blood vessel tissue engineering, cornea regeneration, skin tissue engineering, and periodontal tissue engineering. Furthermore, chitosan can be used as a scaffold material in bone regeneration process, has anti-bacterial effects especially against Phorphyromonas gingivalis, and has low cytotoxicity level when applied to the human body [1,2,3].

Bone is a living tissue composed of 70% minerals and 30% of organic materials, in the form of calcium and phosphate crystals, hydroxyapatite, and several ions such as sodium, fluoride, and magnesium which are constituents of the mineral parts. Thirty percent of the organic materials are mainly composed of collagen fibers and small amount of glycoprotein and proteoglycan [4]. Bone defect is a condition of discontinuity in the structure of bone tissue or cartilage caused by trauma and the bone capable to spontaneously regenerate as before without the formation of scar tissue [5,6]. Data from Riset Kesehatan Dasar (Riskesdas) in 2018 stated that the percentage bone defect cases in Indonesia was 5.5%. Bone defect can heal by producing new bones, but there are complications associated with impaired healing, such as; bleeding, infection, bone strain, hypoxia, and the bone inability to function properly (scar) [7-10].

Currently, the attention is focused on alloplastic bone graft materials, since the gold standard in the bone graft treatment (autogenous bone graft) and allogenous bone graft has the side effect of cell death at the site of the bone donor. The allogenous bone graft process can trigger transmission of infectious diseases carried by the cadaver. The alloplastic material that is currently being widely used is chitosan, the natural biodegradable polymer material which has the ability to act as a scaffold in bone regeneration process through bone graft process.

Search Strategy

A literature search is based on the scientific findings derived from the literature review conducted in January until March 2021 using electronic databases to obtain some information related to the using of chitosan in the relationship with bone regeneration. This study searched each of the databases using the combination of some keywords including Chitosan AND bone regeneration AND bone graft AND scaffold.

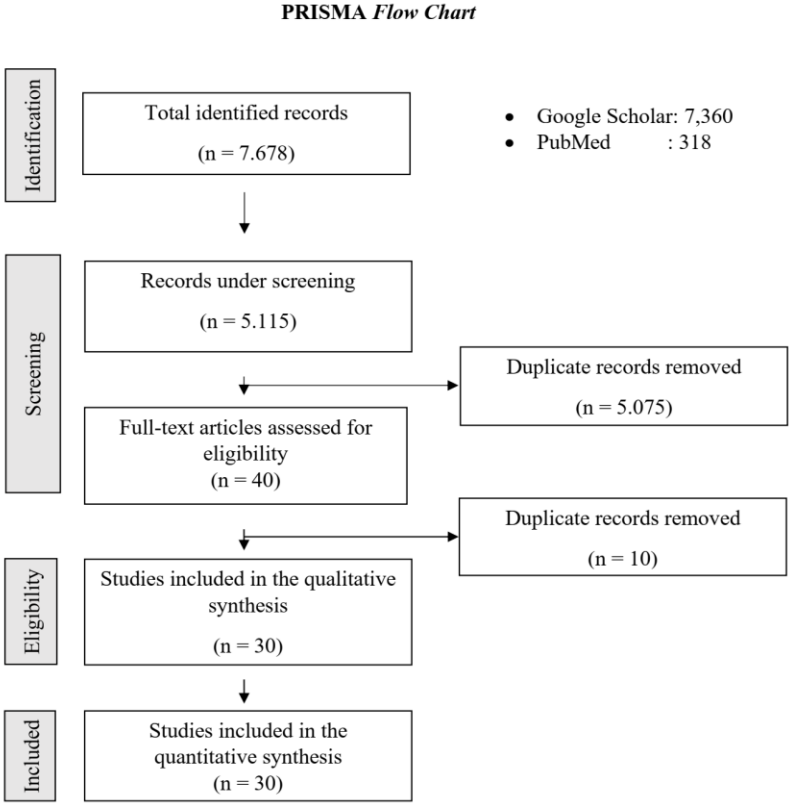

Figure 1. Flow diagram of study identification.

Screening process using PRISMA diagram flowchart

Figure 1 shows the process of writing an article review through the Prisma flow chart**.** The article screening process was carried out based on journal selection criteria, including publications (original articles or review articles) published within the last 10 years, including free journals, journals that have discussions on increasing osteoblasts in bone graft material, Indonesian language journals and or in English. The article screening strategy is carried out through three stages: looking at the article title, abstract article, and full-text article.

Healing

Bone fracture healing consists of two processes, namely: primary/direct bone fracture healing and secondary/ indirectly bone fracture healing. The basic principle that determines the process of the bone healing fracture through directly and indirectly healing is the immobilization level of the fractured bone [11-14].

a. Primary/Direct Healing

The basic principle of primary/ direct bone fracture healing is based on the immobilization level of the fractured bone and will occurs if the fractured bone is fully immobilized [7].

Table 1. Summarizing of Secondary Healing [18-25].

Inflammatory phase	Hematoma	1. Activation of the blood coagulation cascade
		2. Formation of the provisional fibrin matrix
		3. Discharge of harmful signal molecules
		4. Local activation of macrophages
	Acute Inflammation (Acute Phase Response)	1. Recruitment of neutrophils and macrophages
		2. Removal of necrotic tissues and provisional matrix
		3. Production of cytokines, pro-inflammatory chemokines, and growth factors
		4. Recruitment and activation of MSCs, osteoprogenitor, Fibroblast Growth Factor-2 (FGF-2)
	Granulation tissue	1. Active proliferation of progenitor cells
		2. Deposition of immature fibrotic extracellular matrix
		3. The process of angiogenesis
Reparative phase	Callus formation	1. Differentiation of progenitor cells into chondrocytes and production fo fibrin (which occurs in the middle area of the cartilage)
		2. Differentiation of progenitors into osteoblasts and fibrous bone (woven bone) which occurs in the periosteum area.
		3. Factor stabilization by fibrocartilage callus
		4. Apoptosis of chondrocytes and fibrocartilage calcification
		5. Vascular growth and recruitment process of osteoprogenitor cells
		6. Deposition of the fibrous bone (woven bone) on the cartilage scaffold
Remodelling phase	Remodelling	1. Formation of chondroblast and osteoblast
		2. Resorption of cartilage and fibrous bone (woven bone)
		3. Restoration of Harvers system

b. Secondary/Indirect Healing

Bone fracture healing through secondary/indirect pathways occurs when 100% immobilization of the fractured bone cannot be done, or there is micro motion of the fractured bone (summarized in Table 1).

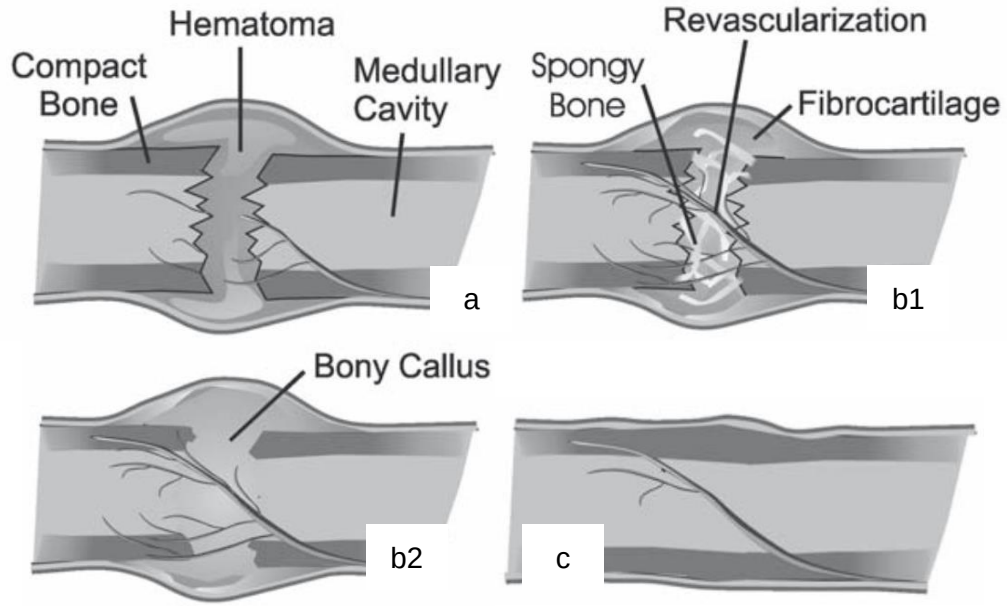

Figure 2. Representation schematic of three stages related to fracture repair [15].

Inflammatory phase

Figure 2a shows the initial response of hematoma occurs due to disruption of local vascularization / discontinuity of blood vessels around the fractured bone area, mainly on the endosteal surface, periosteal surface, within the bone marrow and around the bone's soft tissue. It happens until one week after the bone fracture occurred. Hematoma occurs when the coagulation cascade of plasma is activated and the platelets are exposed to the extravascular environment. The fibrin tissue provides the first provisional matrix which serves as an influx of inflammatory cells drawn by platelets, complement fragments, and harmful signals released by necrotic cells, damaged extracellular matrix cells, and local macrophages [15-19].

Inflammation (Acute Phase Response/APR), Macrophage Survival Phase, happens during the first 24 hours of hematoma, the main inflammatory cells (neutrophils, T cells, B cells, mast cells, macrophages, and eosinophils) play a role in bone fracture healing process, so it is referred as the acute inflammatory process (APR). There are two basic functions of APR in bone fracture healing process, defence function (survival phase) and repair function (repair phase) performed by Classically Activated Macrophages (CAMs) or M1. The former related to the neutrophils that firstly combat in injury and secreted the pro inflammatory cytokine and the macrophage namely macrophage M1. Neutrophils can recruit more monocyte or macrophage cells and prevent the possible of invasion normal flora leading to inflammation. The later related to the polarization from macrophage M1 to the macrophage M2 related to the repair phase through secreting the anti-inflammatory cytokine led to osteoblastogenesis process. [8,20].

Reparative phase

Figure 2b1 and 2b2 show reparative phase lies between the APR phase and the bone remodelling phase. This phase consists of two sub-phases, namely: fibrocartilaginous callus and bonny callus. The former is shown by Figure 2b1 happens in one week until one month and the latter is shown by Figure 2b2 happens in one until four months. The main difference between these two sub-phases is the strength of the formed callus. The callus in the fibrocartilaginous callus is composed of combination between fibrous, cartilage, and chondroblast. The bonny callus is composed of mineralized from the fibrocartilaginous callus. Repair or remodelling phase only occurs when the defence phase has completed its task, which is marked by the establishment of a homeostasis system and the ability to resolve infection. The repair phase is performed by polarization macrophage from macrophage M1 to macrophage M2 that also referred as Alternatively Activated Macrophages (AAMs) which have different function from CAMs. AAMs are more likely to promote collagen's deposition and to restore tissue homeostasis by producing Transforming Growth Factor-β (TGF-β), Interleukin-10 (IL-10), and arginase [15,21].

Table 2. Summarized of anti-inflammatory cytokine related to the bone fracture healing [15].

Transforming growth factor (TGF)-β	• The most potent attractant for macrophage and osteoprogenitor mesenchymal stem cells (MSCs) so it regulates and stimulates the differentiation of osteoprogenitor MSCs into osteoblast
	• The stimulator of angiogenesis
	• Expressed by osteoblast during the ossification procees
	• Relates to the differentiation of fibroblast and deposition of collagen
Bone morphogenetic protein (BMP)	• Induces the differentiation of osteoblast through the RUNX2-mediated
	• It has an osteoinductive property so it stimulates osteoprogenitor cells to proliferate and differentiate into the newly formed bone
Vasculo endothelial growth factor (VEGF)	• Stimulates the differentiation of fibroblast, maturation of collagen, and stimulator angiogenesis
	• Expressed during the reparative phase
Platelet derived growth factor (PDGF)	• Stimulates the differentiation of fibroblast into the maturation of collagen fibre
	• Expressed during the reparative phase
Fibroblast growth factor (FGF)-2	• Induces the differentiation and proliferation of osteoblast through RUNX2-mediated by signaling factor namely PI3K

The pro-inflammatory cytokines and chemokines produced in the repair phase are Transforming Growth Factor-β (TGFβ1, TGFβ2, TGFβ3), Bone Morphogenic Protein (BMP), Vascular Endothelial Growth Factor (VEGF), Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor-2 (FGF-2) which are the key mediators of repair and remodelling process (summarized in Table 2). VEGF produced by cytokines in the repair phase, mesenchymal cells, osteoblasts, and hypertrophic chondrocytes work to repair the supply of periosteal, cortical, and medullary due to fracture, which triggers cellular and local acidosis so it needs revascularization. VEGF acts as angiogenesis and vasculogenesis, angiogenesis is the process of new blood vessels formation from pre-existing blood vessel branches whereas vasculogenesis is the process of blood vessel formation in de novo from endothelial progenitor cells (EPCs) in situ inside callus.

Remodelling phase

Figure 2c shows bone remodelling comprised of four phases that take 3-6 months [3,26]:

1. Activation phase
   The activation phase is triggered by mechanical stress and bone nutrition (apart from being caused by parathyroid hormone and estrogen) the key of activation phase initiation is terminally differentiated osteocyte existence.
2. Bone resorption phase
   The bone resorption phase occurs within 8-10 days and initiated by organic matrix break-down by osteoclasts (multinucleated) in the old bone.
3. Reversal phase
   The reversal phase connects the bone resorption occurred by osteoclast with bone formation occurred by osteoblast, this phase lasted 7-14 days. There are four different types of osteoclasts in the reversal phase; osteoclast cells matrix-derived factor released during bone resorption process, factors secreted by osteoclasts (cardiotrophin-1, spingoshine-1-phosphate, collagen triple helix, and complement type 3a, factor membrane bound osteoclasts (Ephrin B2, semaphoring D), and structural change factors by osteoclasts on the surface of bone tissue. Reversal cells are the cells derived from pre-osteoblast that colonize on the surface bone eroded by osteoclast and respond to osteoclasts and coupling factors similar to fibroblast cells covering the surface of the bone.
4. Bone Formation Phase
   The bone formation phase begins with mononucleated osteoblast cells synthesizing new bone matrix from collagen fibres and non-collagen proteins, which is primarily deposited as calcium hydroxyapatite.

Briefly, the remodelling phase related to the three cells, namely: osteoclast, osteoblast, and osteocyte. Osteoblast is derived from osteoprogenitor of mesenchymal stem cells and deposits mineral extracellular matrix. Osteoclast is giant cell that resorbs bone. Osteoblast and osteoclast balance are determined through bone remodelling through RANK/RANKL/OPG. RANK will bind to RANKL and the OPG is the decoy receptor to prevent the binding of RANK and RANKL.

Bone graft material consists of four types; autogenous bone graft (Autograft), allogenic bone graft, xenogeneic bone graft, and alloplastic graft materials. The criteria for bone graft material are osseointegration, osteoconduction, osteoinduction, and osteogenesis. Osseointegration is the ability to bind chemically to the bone surface without causing scarring, osteoinduction is the ability to induce pluripotent stem cells from the surrounding tissue to become osteoblasts, osteogenesis is the ability to directly produce new bone from osteoblast cells. The gold standard bone graft material is autogenous bone graft, but there are several drawbacks, one of which is death in the area where the graft material was sampled. Whereas allogenic materials disadvantage is a risk of disease transfer from the donor to the recipient.

Chitosan in Bone Regeneration Therapeutics

Figure 3 explains about the concept mapping of chitosan act as anti-bacterial due to its cationic properties, ease of penetration into bacterial nucleus, and the chelating process of the material.

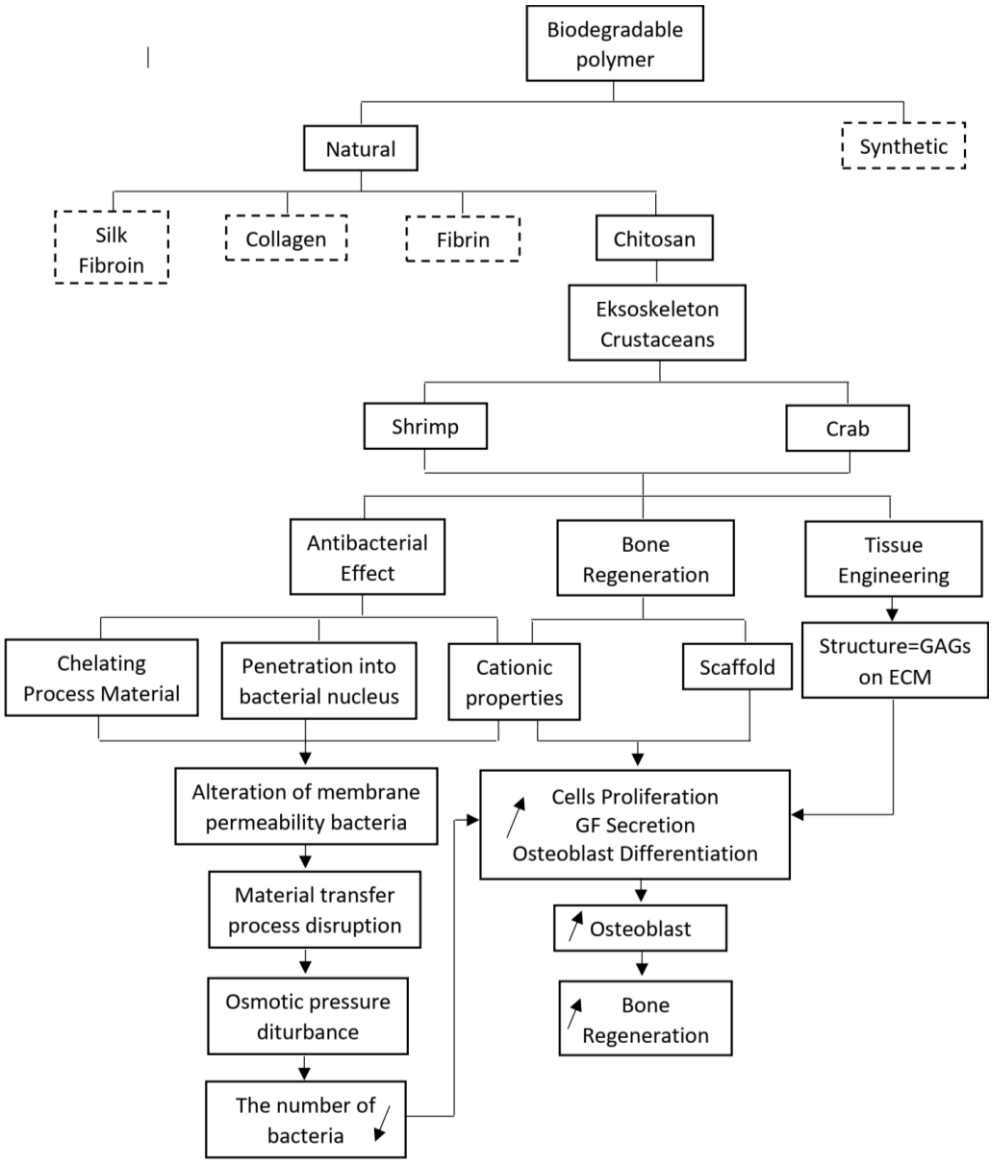

Figure 3. Chitosan as Scaffold Material Bone Graft.

Cationic properties of chitosan in consequence of an amine group (R-N) (CH3) 3+ on chitosan structure allows it to interact with negative properties of the bacterial cell membrane. The interaction between chitosan and bacterial cell membranes is a strong electrostatic interaction, causes changes in the permeability of the bacterial cell membranes and disruption of the material transfer process. As the material transfer process is disrupted, the osmotic pressure increases, causing bacteria to lyse. Bacterial lysis is characterized by the loss of protein fluid and intracellular material which leads to bacterial death. The second mechanism as an anti-bacterial agent is manipulated by the ability of chitosan to penetrate into the bacterial cell nucleus and bind to bacterial DNA, causes inhibition of mRNA and protein synthesis. Furthermore, chitosan bonds with bacterial DNA also intervene in the energy metabolic processes associated with bacterial death. The third mechanism is the ability of chitosan to inhibit and kill bacteria due to the metal chelating process. Chitosan molecules that surround bacteria can form complex shapes with metals and inhibit the flow of nutrients that are important to bacteria, causing the bacteria to die [3].

Cationic properties, which are related to bacterial inhibition, are also associated with increased adhesion, proliferation, differentiation and mineralization of osteoblasts. Chitosan bioactive activity is characterized by its osteoconductive, osteoproductive, and osteoinductive properties. Simply put, the cationic properties of chitosan make it possible to combine it with anions regulating growth factors and cell activity. Hence, chitosan's cationic properties allow it to be combined with anions that regulate growth factors and cell activity, also the ability of chitosan as a scaffold material related to the use of scaffold nanocomposites made of Chitosan and Hydroxyapatite, in which the two substances form cross-linked bonds [27-30].

All of these three phases in bone regeneration namely inflammatory, reparative, and remodeling phase need supporting from the chitosan. Chitosan as an anti-bacterial agent has benefit in the bone regeneration process, especially when in inflammatory phase. In inflammatory phase, chitosan can act as an antibacterial agent from amine group so that it can prevent the invasion of normal bacterial or inhibit the number of bacterial invasions. Because of this, the macrophage M1 will soon to polarize to macrophage M2. This polarization relates to the changing of pro-inflammatory to anti-inflammatory macrophage. The anti-inflammatory macrophage, then relates to the secretion of the number of anti-inflammatory cytokines that relates to the osteoblastogenesis in the repair phase. Osteoblastogenesis is related to the property of the bone graft inserted namely: osteoconductive, osteoinductive, and osteogenesis. The acceleration of repair phase will lead to the acceleration of remodeling phase. All of these properties then related to the deposition and mineralization of extracellular matrix then leading to the maturation of osteoblast through the ossification process.

Conclusions

The present review shows that Chitosan has so many effects in bone regeneration process, such as: antibacterial property and bioactive activity which can be used both as a bone scaffold and antibacterial agent in bone regeneration. This antibacterial property then will accelerate the polarization of macrophage M1 to be macrophage M2. The macrophage M2 then secretes the anti-inflammatory sitokine that relates to the osteoblastogenesis process. This process relates to the deposition and mineralization of extracellular matrix leading to the maturation of osteoblast become osteocyte called ossification process.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the manuscript.

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