UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO:

Effects of different external fields on collagen piezoelectricity and bone tissue mineralization

Trabajo de titulación presentado como requisito para la obtención del título de Ingeniera Biomédica

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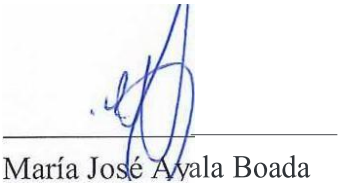

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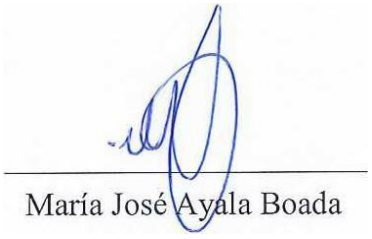

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DEDICATORY

To my beloved Babe and Peach

I dedicate this degree project to all the women who have been part of my life, starting with the women in my family, my mother, aunts, and cousins who have supported me during my university career. To my friends, who have become my support system, I have always been able to find help from them and all the sorority women I have met and that I have shared moments with.

Likewise, I dedicate this work to all the girls and women who have been victims of femicide and abuse; we want each other alive to follow our dreams, we want each other alive to fight for those who are no longer, for them, with all my love.

María José Ayala Boada

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I thank those people I met during the process of carrying out this work for encouraging me to finish it.

María José Ayala Boada

RESUMEN

El sistema óseo está formado por órganos llamados huesos que cumplen múltiples funciones. Estas incluyen servir como soporte para los otros órganos del cuerpo y permitir el movimiento. Al ser órganos complejos, comprender cada uno de sus procesos para mejorarlos mediante técnicas innovadoras ha sido objeto de estudio durante décadas.

El hueso consta de tejido óseo formado por células que componen el 2% y matriz extracelular, que constituye el 98%. La interacción entre las células y la matriz forma la base para la remodelación ósea. Este proceso ocurre constantemente donde ocurren las microfracturas, las cuales, a su vez, producen pequeñas cargas eléctricas endógenas que activan las funciones de los osteoblastos y osteoclastos mediante la segregación de hormonas y factores de crecimiento. Cuando este proceso está en equilibrio, el resultado es nuevo tejido óseo; sin embargo, una descompensación en el proceso provoca debilitamiento y reducción de la masa ósea.

La matriz extracelular está constituida por la parte orgánica, mayoritariamente formada por colágeno, una proteína cuaternaria que se le atribuye la característica de la piezoelectricidad gracias a su disposición en forma de bandas estriadas; y la parte inorgánica que mineraliza el colágeno a través de cristales de hidroxiapatita. Esta combinación le da al hueso flexibilidad y resistencia para poder cumplir con sus funciones. La piezoelectricidaddel hueso causada por corrientes eléctricas endógenas es responsable de la remodelación ósea; por lo tanto, siguiendo este principio, la remodelación puede inducirse a través de campos externos.

La presente revisión literaria expone las estructuras, características y procesos del hueso y el tejido óseo y los efectos de los campos mecánicos, eléctricos y magnéticos externos sobre la remineralización del tejido óseo y la piezoelectricidad del colágeno.

Palabras clave: Piezoelectricidad del colágeno, mineralización ósea, campo eléctrico, campo magnético, campo mecánico.

ABSTRACT

The bone system is made up of organs called bones that serve multiple functions. These include serving as support for the other organs of the body and allowing movement. Being complex organs, understanding each of their processes to improve them through innovative techniques has been the object of study for decades.

Bone consists of bone tissue made up of cells that make up 2% and extracellular matrix, which makes up 98%. The interaction between cells and the matrix forms the basis for bone remodeling. This process constantly occurs where microfractures occur, which, in turn, produce small endogenous electrical charges that activate osteoblast and osteoclast functions through the secretion of hormones and growth factors. When this process is in balance, the result is new bone tissue; however, decompensation causes weakening and reduction of bone mass.

The extracellular matrix comprises the organic part, mostly made up of collagen, a quaternary protein attributed to the characteristic of piezoelectricity thanks to its arrangement in the form of striated bands, and the inorganic part mineralizes collagen through hydroxyapatite crystals. This combination gives the bone flexibility and resistance to be able to fulfill its functions. Furthermore, the piezoelectricity of the bone caused by endogenous electrical currents is responsible for bone remodeling; therefore, following this principle, remodeling can be induced through external fields.

This literary review exposes the structures, characteristics, and processes of bone and bone tissue and the effects of external mechanical, electrical and magnetic fields on bone tissue's remineralization and collagen's piezoelectricity.

Keywords: Collagen piezoelectricity, bone mineralization, electric field, magnetic field, mechanical field.

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1. INTRODUCTION

The application of external fields in bone mineralization has been under investigation since (Fukada & Yasuda, 1957) discovered bone piezoelectricity. A property attributed to collagen molecules allows electrical charges to arise under mechanical stress or an applied external field in the form of activation signals for bone remineralization.

Bones are constantly undergoing microscopic fractures from the stress of daily activities such as walking. They do not cause any damage or represent any problem because they are quickly repaired thanks to small but powerful electric fields generated by the bones matrix; the repair occurs thanks to the osteoblasts detecting the effects of the fields and repairing the tissue.

It has been possible to understand the different variables that influence the process mentioned above and develop sophisticated medical equipment to improve treatments. Since then, research on electric fields, magnetic fields, and mechanical forces applied individually has not stopped. However, there is still no study where these three external fields are compared according to established variables.

For this reason, this literary review makes a study of the effects of external electric and magnetic fields and mechanical forces, depending on the piezoelectricity of collagen and bone remineralization, taking as variables to compare magnitudes, frequencies, and application methods.

Chapter two presents a theoretical review of bone composition, processes, and properties, taking into account piezoelectricity. Chapter three presents a literary review about studies and research in applying external fields for tissue remineralization. Finally,

a discussion and conclusions are presented where each technique's advantages and disadvantages are detailed and future applications.

1.1Objectives

General

- Carry out a literary review about the influence of external fields on the remineralization of bone tissue and piezoelectricity of collagen

Specific

- Compare studies performed using external mechanical field in bone tissue remineralization.
- Compare studies performed using external electrical field in bone tissue remineralization.
- Compare studies performed using an external magnetic field in bone tissue remineralization.

2. THEORETICAL BACKGROUND

Collagen is a complex protein formed by the union of triplets of amino acids and is the most significant organic part of bone tissue (Brodsky & Ramshaw JAM, 1997). Thanks to its characteristics, collagen is responsible for the bone piezoelectric effect, which, in turn, is responsible for the re-formation of the bone matrix in response to a stimulus.

This chapter explains the structure, synthesis, and types of proteins and collagen. It also explains the structure, remodeling, regeneration, and piezoelectric effect of bones andbone tissue.

2.1 Aminoacids

The amino acids (AAs) are organic molecules that in their structure present an amine group ($-NH_2$) at one end and a carboxyl group (-COOH) at the opposite end (Brodsky & Ramshaw JAM, 1997) (see Figure 1). The two AAs combination forms a peptide bond due to the condensation reaction between them (Duarte Quintanar, 2006), and the product is called a dipeptide; when a third amino acid joins, it is called a tripeptide, and consequently, a polypeptide is formed (Duarte Quintanar, 2006). When the peptides form a polypeptide chain and reach a defined molecular weight, it is called a protein (Gelse et al., 2003).

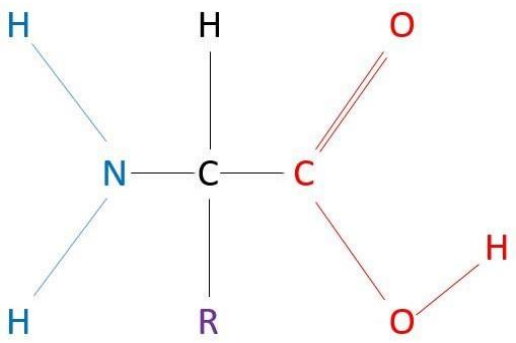

Figure 1. Aminoacid general structure

2.2 Proteins

Proteins are polypeptides made up of long chains of amino acids, which correspond to the gene's DNA sequence that encodes it. The main characteristic of the proteins is to have a defined stable three-dimensional structure (Brocchieri & Karlin, 2005, p.3390).

2.2.1. Protein biosynthesis

Protein biosynthesis is an overly complex process carried out by ribosomes and guided by the information of a messenger RNA ($_m$RNA) molecule that acts as a template. (D. L. Nelson et al., 2005).

Proteins are assembled from their amino acids using information encoded in genes. Each protein has its amino acid sequence specified by the nucleotide sequence of the gene that encodes it. The genetic code is made up of a set of three nucleotides called codons, and each codon designates an amino acid (D. L. Nelson et al., 2005).

Genes encoded in DNA are first transcribed into pre-messenger RNA using various forms of post-transcriptional modification to form mature $_m$RNA, which is used as a template for protein synthesis on the ribosome. The $_m$RNA is loaded onto the ribosome and read by pairing each codon with its complementary anticodon located on a transfer RNA ($_t$RNA) molecule that carries the amino acid corresponding to the codon it recognizes. The growing polypeptide is called the nascent chain (De Robertis et al., 2012).

Proteins always biosynthesize from the N-terminus to the C-terminus. In this way, the protein's primary structure is achieved its amino acid sequence. Now it must be folded properly way to reach its native structure, which performs the function (De Robertis et al., 2012).

2.2.2. Protein structure

Proteins are organized in a certain way to acquire their structure. Suppose the conditions such as temperature or pH change, the protein loses its shape and function by denaturation (De Robertis et al., 2012). Once the denaturant is removed, most proteins cannot be refolded.

According to the polypeptide chains' weight and shape, there are four hierarchical protein structure, as can be seen in Figure 2 (Brodsky & Ramshaw JAM, 1997).

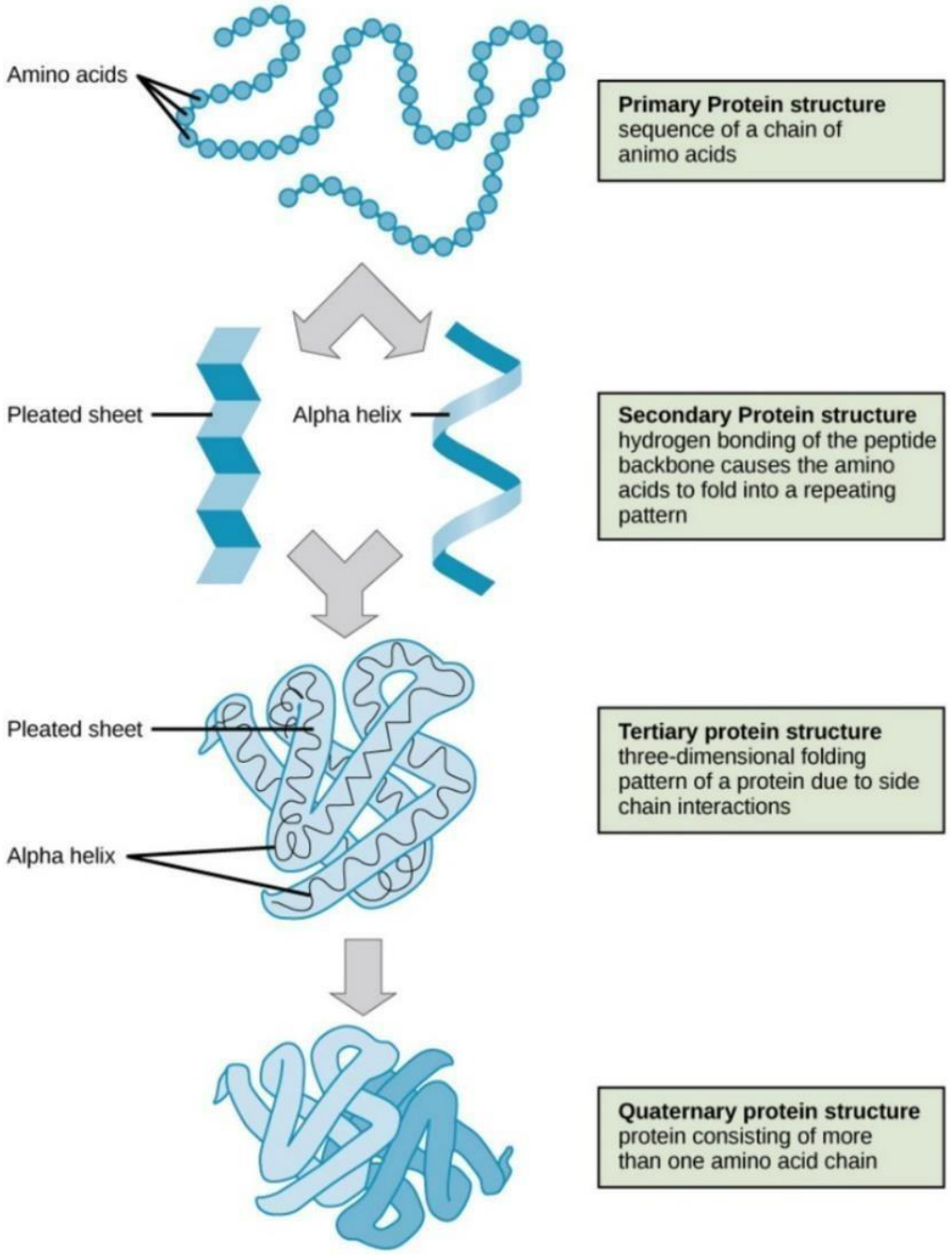

Figure 2. The hierarchical structure of proteins. By (Daudert, 2018)

Collagen is a quaternary protein constituted by three polypeptide chains that roll in a left-handed way (levogyre) on itself. As the 3- α-helix fold forms the triple collagen helix, a right-handed structure (dextrogyre) is formed (Devlin, 2004).

2.3 Collagen

Collagen is the most abundant protein in mammals and the main structural protein in the extracellular matrix in various connective tissues of the body, making up from 25% to 35% of the whole-body protein content (Ozawa et al., 2008). It consists of AAs bounds together to form a triple helix of elongated fibril known as collagen helix, characterized by its resistance (a fiber of 1 mm diameter can support a load from 10-40 kg) (Prockop & Guzmán, 1981).

It can be found in tendons, ligaments, and skin. By the degree of mineralization, collagen tissues may be rigid (bone), compliant (tendon), or have a gradient from rigid to compliant (cartilage). It is also abundant in corneas, blood vessels, the gut, vertebral discs, and the dentin in teeth.

2.3.1 Collagen biosynthesis

The biosynthesis of collagen is an elaborated multistep process (Gelse et al., 2003) that begins with gene transcription within the nucleus to the aggregation of collagen heterotrimers into large fibrils.

The initial phase begins when the polyribosomes of the rough endoplasmic reticulum (RER) read the $_{m}$RNA and then assemble AAs to form the polypeptide chains. These chains are the pro- α chains and are precursors for α –chains, carry supplementary AA sequences at their ends. The pro- α chains will undergo hydroxylation within the ER. Subsequently, galactose and glucose molecules are attached to the hydroxylated groups, while other sugars are attached to the chains' terminal groups. Finally, disulfide bridges are created between the polypeptide chains, leading to the pro-collagen molecule formation (Prockop & Guzmán, 1981).

Depending on the end where the pro-collagen molecule is located, there are N-protease (PINP) or C-protease (PICP), which prevented pro-collagen from early assembling itself inside the cell (see Figure 3). Then, it transmits through the Golgi vesicles and passes into the extracellular matrix in which, under the action of proteases, it undergoes cleavage of the propeptides. After this cleavage, the collagen molecules become fibers (Prockop & Guzmán, 1981).

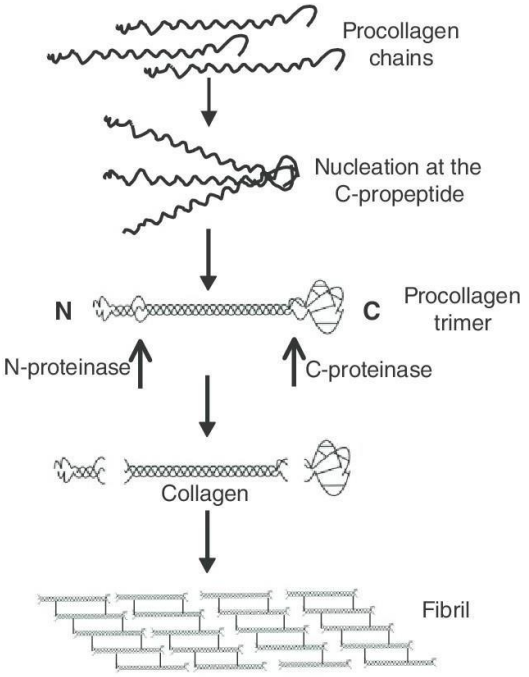

Figure 3. Overview of the steps involved in the production of collagen fibrils by fibroblasts.

By (Haba et al., 2017)

Finally, different collagen molecules are associated with forming bands through the telopeptides at the ends, and in turn, covalent bonds are established. The creation of cross-links between the polypeptide chains ensured the great strength of the molecule.

2.3.2. Collagen structure

2.3.2.1. Primary structure

It is also called the aminoacids triplet. The primary structure is a unique sequence of proline-rich Gly-X-Y polypeptide linked together by peptide bonds with two main characteristics.

a) Glycine (Gly) makes up 30% of the entire length of the ~1000 amino acids in each chain with the strict repeating sequence – (Gly-X-Y) $_n$-. The repetitions linked together formed the α -chains (Persikov et al., 2005).

b) In most cases, proline (X) and hydroxyproline or hydroxylysine (Y) form residues. While in other proteins, hydroxyproline is not commonly found, in collagen, it constitutes more than 50% of the total AA content (Ferreira et al., 2012).

2.3.2.2. Secondary structure

The α-chains build the triple helix of type I, II, and III collagens, a levogyre helix that is not stabilized by hydrogen bridges because it has a tiny diameter (0,5 nm) (Brodsky & Ramshaw JAM, 1997).

The non-helical domains are the C-terminus involved in the initiation of triple-helix formation and the N-terminus involved in regulating primary fibril diameters (Brasselet et al., 2005).

2.3.2.3. Tertiary structure

It is also known as the triple helix. The α -chains are linked to each other by hydrogen bonds forming a triple helix right-handed stabilized by hydrogen bonds

and other covalent bonds (Gelse et al., 2003). In other words, the triple helix of collagen is a dextrogyre helix formed by 3-α levogyre helices.

2.3.2.4. Quaternary structure

Collagen molecules can self-assemble into a supramolecular form which constitutes five triple-helical molecules called collagen fibers (Watson et al., 2014), as shown in Figure 4.

Tropocollagen is the basic unit of collagen fiber, and its repetition forms the organized fibers in parallel rows. In each row, the tropocollagen units are highly oriented with D-periodic banding spaces, where D is ~67nm and are ¼ of their length ahead of the next row so that every four rows (the 1st and 5th row) coincide with the tropocollagen units at the same height giving the characteristic striated appearance (Prockop & Guzmán, 1981).

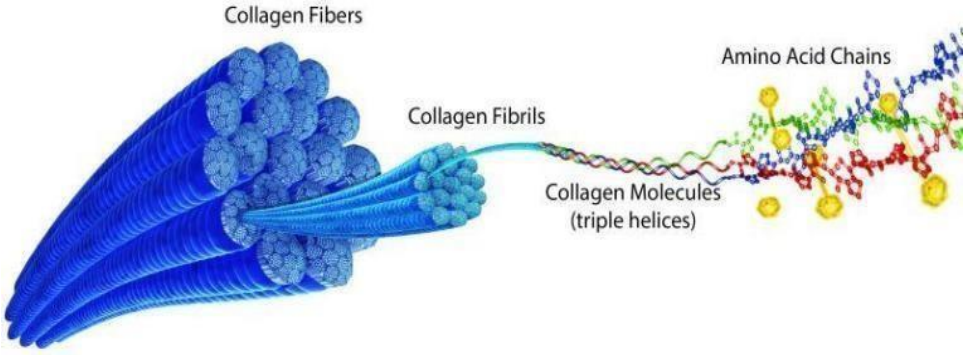

Figure 4. The basic structure of collagen type I. By (Rossert & de Crombrugghe, 2002)

2.3.3. Collagen types

Instead of being a single protein, collagen is considered a family of closely related but genetically distinct molecules. There are 21 types of collagen, the first five being the most important:

Type I: Skin, tendon, organs, **bone** (the main component of the organic part of the bone).

Type II: Cartilage (a main collagenous component of cartilage).

Type III: Reticulate (the main component of reticular fibers).

Type IV: Forms basal lamina, the epithelium-secreted layer of the basement membrane.

Type V: Cell surfaces, hair, placenta, and bone. (Fratzl, 2003).

Collagen type I is usually formed as a heterodimer of two identical α_1(I) - and α_1 (II)-chains and one α_2 (I)-chain with about 1000 AAs, has a length (L) approximately of 300 nm and a diameter of 1,5 nm (Nudelman et al., 2013). Fibroblasts, chondroblasts, and osteoblasts synthesize collagen, and their primary function is resistance to stretching.

2.3.4. Collagen properties

The striated appearance of collagen fibers is a key to the existence of a potential difference between each tropocollagen resulting in a direct piezoelectric effect (Shamos & Lavine, 1967).

An essential characteristic of collagen is the anisotropy, that is, its properties (conductivity, elasticity, piezoelectricity, temperature, and more) and its mechanical behavior change depending on the angle where the sample was taken (Fukada & Yasuda, 1964), even though it is taken from the same bone matrix.

Understanding this characteristic has been fundamental in determining the processes that occur when the bone is subjected to stress. It was taken to consider the experiments carried out by Fukada & Yasuda during 1954-1957. They took the femur as an example

and concluded a difference between its biological and mechanical axis due to its skeleton position. Using X-ray diffraction, it was found that the axis of symmetry of collagen in the human femur is displaced from the axis of the bone, which produces a direct piezoelectric effect. (Fukada & Yasuda, 1957).

Besides, like many other crystals, collagen is not centrosymmetric. Thus, the combination of stresses can exist that separates the gravity centers of their positive and negative charges to produce an induced dipole moment.

2.4. Bones and bone tissue

Bones are living and rigid organs that form the skeleton, fulfill functions such as support and protection, make movements possible by serving as a place of incision to the muscles and produce cells that are part of the blood in a process called hematopoiesis (Tortora & Derrickson, 2013).

2.4.1. Bone properties

Bone has the following mechanical properties:

Anisotropic structure: The bone shows different mechanical characteristics when the load is applied in different directions.

Porous material: Porosity varies from the cortical bone (5%) to the cancellous (90% porosity) (Vannessa & Cerrolaza, 2013).

Viscoelastic behavior: Living bone is both elastic and viscous, providing excellent resistance to excessive efforts. Furthermore, in the dry state lose its property, this characteristic allows better resistance to fast efforts than slow ones.

Figure 4 shows an example of a force-displacement curve. Within a range of loads, the bone presents elastic properties because when the load ceases, the bone returns

to its original size and shape, yet, above an absolute magnitude of stress, known as the yield point, the bone presents plastic properties as it is permanently deformed, and if the stress increases, it reaches the fracture point. (Pearson & Lieberman, 2004).

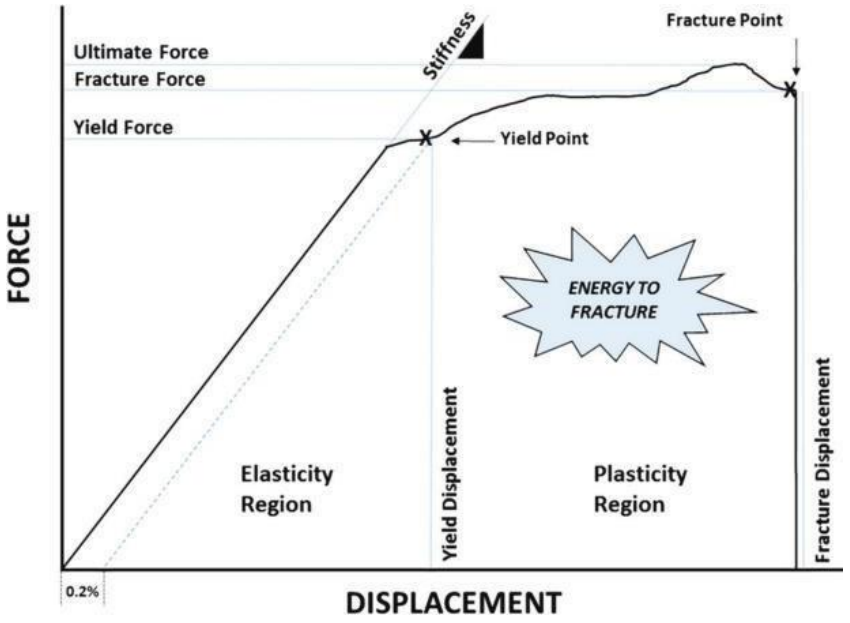

Figure 5. Force- displacement curve. Demonstrate that bone presents elastic properties before the yield point and plastic properties after the yield point. Taken from (Hart et al., 2017)

2.4.2. Bone structure

Bone compact or cortical bone, cancellous or trabecular bone, and bone marrow make up the bone structure (see Figure 6). Cortical lies outside of all bones and on the diaphysis of long bones, while trabecular is found mainly in the epiphyses of long bones, inside of short bones, and contains bone marrow (Tim, 2018).

Bone marrow is found in the center of most bones and has many blood vessels. There are two types of bone marrow: red and yellow. Red marrow contains blood stem cells that can become red blood cells, white blood cells, platelets, and yellow marrow made mostly of fat (Safadi et al., 2009).

Bones are a complex structure composed of 25% organic matrix (Collagen), 65% of an inorganic matrix (Hydroxyapatite), and 10% water (Sulca Buitrón, 2019).

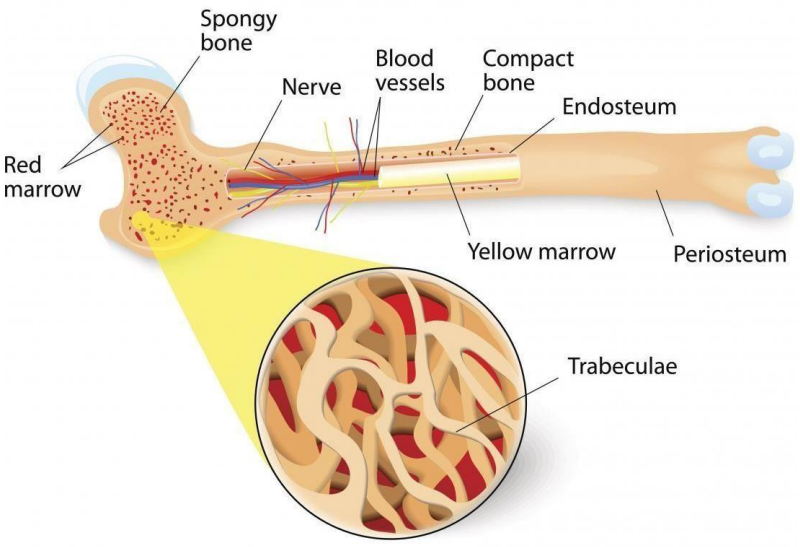

Figure 6. The bone structure. By (Tim, 2018)

2.4.2.1. Bone tissue

The bone tissue is a specialized connective, hard, firm, and resistant tissue composed of cells and an extracellular matrix. The cells represent 2%, while the extracellular matrix is 98% of the bone tissue.

2.4.2.1.1. Bone cells

Osteoblasts are a general class of mesenchymal cells that originate from mesenchymal precursor cells in various tissues, including the periosteum and endosteum. It forms bone by synthesizing collagen matrix and then secreting calcium-phosphate mineral (Serrano, 1998).

The endocrine system regulates the osteoblast function, including parathyroid hormone, vitamin D, calcitonin, and sex steroids (Sánchez Cruz et al.,

2006). Additionally, estrogen plays a significant role because it regulates osteoblast (and chondroblast) activity (Osuna, 2003).

Osteoclasts are formed by the fusion of several mononuclear cells derived from a blood stem cell from the red marrow. They are multinucleated and giant cells that degrade, reabsorb, and remodel bones. Osteoclasts occupy a cavity called the resorption lagoon or Howship lagoon and are located on the bone surfaces firmly associated with the bone matrix, through integrins (α5β3) (Väänänen et al., 2000).

Osteocytes are formed from osteoblasts when they become entrapped in the osteoid matrix. They are the primary cells of bone tissue, representing approximately 95% of the total tissue cells. Each osteocyte is located in a gap carved into the mineralized intercellular substance of the bone. It has a characteristic stellate shape because of its cytoplasm, some extensions that run along small channels located in the bone's mineralized matrix, forming a labyrinth of ducts lacuna-canalicular system (Bellido & Pellegrini, 2016). Osteocytes can segregate or reabsorb the matrix bone that surrounds them.

Osteogenic cells are stem cells that divide to produce osteoblasts and assist in repairing bone fractures. The overproduction of osteogenic cells causes a type of cancer called a sarcoma (Niggemeyer, 2018).

Figure 7 shows the type of cells in bone tissue.

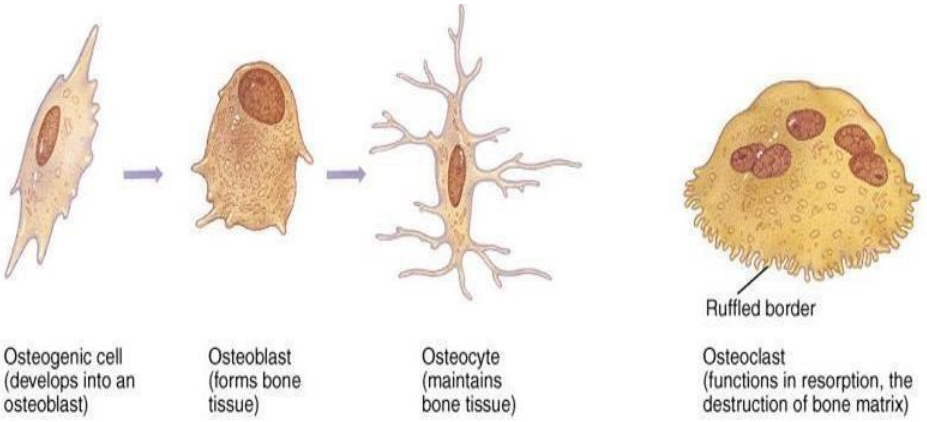

Figure 7. Type of cells in bone tissue. By (Niggemeyer, 2018)

2.4.2.1.2. Extracellular matrix

The extracellular matrix is made up of an organic and inorganic phase. The organic phase contains proteoglycans (but less than cartilage), glycosaminoglycans, glycoproteins, osteonectin, osteocalcin, and collagen fibers (mainly type I (90%), with some type V) (Peckham et al., 2003). On the contrary the inorganic phase is rich in Ca^{+2} and P^{-3} and comprise a hydroxyapatite bone mineral. (Nordin & Frankel, 2004).

2.4.2.1.2.1. Organic phase

The organic phase comprises collagen fibers of type I and V forming several collagen fibers, known as tropocollagen (Ozawa et al., 2008).

The second component is osteonectin, a protein that can interact with collagen and salts located in the most calcified areas. Other proteins are osteopontins that bind hydroxyapatite, bone morphogenetic proteins (BMPs), and acid proteoglycans (Ozawa et al., 2008).

Thanks to collagen's characteristics and properties, the organic matrix provides flexibility and resistance to bone tension. The lack or deformity of them

can cause diseases such as ontogenetic imperfection or crystal bones.

2.4.2.1.2.2. Inorganic phase

The inorganic phase contains plenty of crystallized mineral salts in the form of small hydroxyapatite (HA) crystals ($Ca_{10}(PO_4)_6(OH)_2$) and small amounts of various impurities such as nitrate, fluoride, and magnesium. These salts are deposited in a reticular matrix formed by collagen fibers through a process known as mineralization (Gonz et al., 2016).

The hydroxyapatite has a small, ribbon-like structure (~ 25 nm wide, 10 nm high, and 50 nm long) (Ozawa et al., 2008). Allowing its formation and growth time to be shorter than that of collagen facilitates bone development. Embryonic, bone repair/regeneration, and efficient osteoclast function during bone remodeling. Figure 7 summarizes bone and bone tissue structure from a macro to nanoscale.

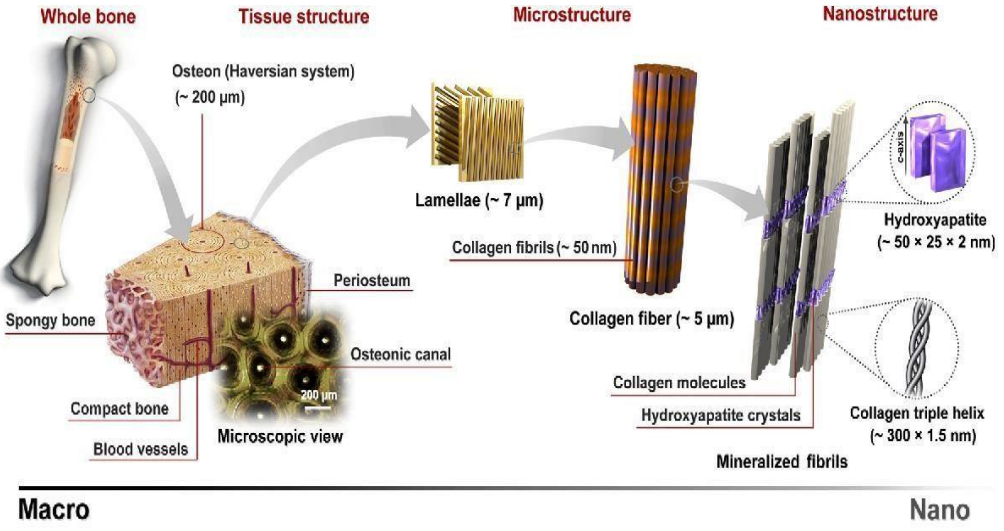

Figure 8. The hierarchical structure of typical bone at various length scales. The microstructure of cortical or compact bone consists of Haversian systems, and at the nanoscale, the structural framework is collagen fibers composed of bundles of mineralized collagen fibril. By (Sadat-Shojai et al., 2013)

2.4.3. Bone formation

Ossification or osteogenesis is creating new bone tissue by osteoblasts, and it is divided into two types: membranous or endochondral and intramembranous (Caetano-Lopes et al., 2007).

Bone formation begins at six weeks of fetal development, and although the exact process is unknown, it is attributed to various growth factors and cytokines.

In long bone fractures treated with plaster, endochondral osteogenesis is the most common, while in open fractures, the bone is exposed and stabilized with plates and metal screws, and intramembranous osteogenesis is the most common.

2.4.3.1. Membranous Ossification

Developed during fetal formation due to the progressive hardenings of hyaline cartilage until it becomes hard tissue (Castillo Gutiérrez, 2017). It is essential in forming long bones such as the appendicular and axial skeleton and the mandible.

The process, also known *as endochondral ossification* begins "from the inside to the outside"; the reverse process "from the outside to the inside" forms perichondrium by layer cells coating bone externally (Peinado et al., 2009).

General Process:

1. The perichondrium becomes periosteum as the perichondral cells become osteogenic cells and deposit a layer and sub-layer of cells, forming a perichondral or ossification ring around the central zone hyaline cartilage.
2. The perichondral ring externally surrounds the growth plate and consists of three regions where osteoblasts and osteoclasts act (Peinado et al., 2009). The fibrous region has parallel bundles of collagen fibers where the fibroblasts arrange.

2.4.3.2. Intramembranous Ossification

Developed during fetal formation within the periosteum; it is also reabsorbed, allowing remodeling (Calixto et al., 2013). Intramembranous ossification is essential during the natural regeneration of bone fractures and the formation of the human head's bones (Kiss & Szentágothai, 1987).

General process:

1. The mesenchymal cells present in the membrane and become osteochondral progenitor cells that will become osteoblasts.
2. Osteoblasts produce bone matrix and are enveloped by collagen fibers, turning them into osteocytes.
3. Trabeculae are formed, which are subsequently trapped to produce bone.
4. The trabeculae will unite to produce spongy cells that will specialize in producing red bone marrow (Calixto et al., 2012).
5. From the periosteum that surrounds the developing bones, the bone matrix's osteoblasts will produce compact bone.

2.4.3.3. Heterotopic Ossification

Although it is not part of the ossification process, it is defined as the growth of mature bone in the soft tissues adjacent to previously injured areas (Chaverri Fierro & Abellán Miralles, 2016). The heterotopic ossification can occur anywhere on the body, but the hips, knees, shoulders, and elbows are the most common bone sites. The risk factors such as brain or spinal injuries, bone surgeries, burns, infections, or trauma increase its incidence.

2.4.4. Bone remodeling

Bone remodeling is the process that bone undergoes during the vertebrate's life. In the human species, the process occurs until the age of 30 when the maximum bone mass is reached, which remains relatively stable until the age of 50; it begins to decrease until death (Fernandez-Tresguerres Hernandez-Gil et al., 2006).

Notwithstanding that bone remodeling's initiation stimulus has not yet been identified, evidence shows that it can be initiated by mechanical stress resulting from physical exercise, growth, or during mechanical loading (Rudnicki et al., 1993).

The bone is a dynamic organ transforming itself, replacing the old tissue by the osteoclasts with new tissue by osteoblasts' function. In the formation process, osteoblasts synthesize a new bone matrix that hydroxyapatite crystals will later mineralize. The dynamism between bone resorption and formation is kept in balance through biochemical cascades regulated by growth factors and hormones detailed in Table 1 and Table 2, respectively.

Table 1. Hormones that regulate bone remodeling. By Meghji (1992)

Polypeptide hormones
Parathyroid hormone
Insulin
Calcitonin
Growth hormone
Steroid hormones
Glucocorticoids
Sex steroids
1,25-dihydroxyvitamin D3
Thyroid hormones

Table 2. Growth factors that regulate bone remodeling. By Meghji (1992)

Insulin-like growth factors (IGF) I and II
Selected cytokines of the interleukin (IL), tumor necrosis factor (TNF), and colony-stimulating factor (CSF) families.
Fibroblast growth factors (FGF)
Platelet-derived growth factors (PDGF)
Transforming growth factors-β (TGF-β) superfamily, including the bone morphogenetic proteins (BMPs)

The growth factors are polypeptides found in bone and non-bone tissue; they fulfill important functions in the replication and differentiation in cell growth of both cells of the same class (autocrine factors) and cells of another class within the same tissue (paracrine factors) for which they are essential in the process of bone remodeling and possibly an anomaly can cause bone disorders (Hill, 1998).

At the microscopic level, bone remodeling occurs in Basic Mineral Units (BMUs), located on the trabecular surface. BMUs have a half-life between 2 to 8 months and are defined as a pool of pre-osteoblasts, osteoblasts, macrophages, and osteoclasts (Fernandez-Tresguerres Hernandez-Gil et al., 2006). Each BMU is separated from the other, which suggests that its activation is controlled locally, and it is hypothesized that it may be due to paracrine or autocrine factors generated in the bone microenvironment. (Hill, 1998).

Every ten years, the skeleton is completely renewed, and although it is a process that occurs for life, it can suffer abnormalities that trigger pathologies such as osteoporosis (deficiency) and osteopetrosis (overproduction).

Bone remodeling occurs in 5 phases that are detailed below:

1) Resting Phase

It is also called a quiescent phase. Bone is not dividing.

2) Activation phase

The activation phase begins with the maturation of pre-osteoclasts to mature osteoclasts. The lining cells are found on the edges of bone, capturing stimuli and secrete the RANKL (Receptor Activator for Nuclear Factor κ B Ligand) ligand-protein activates its respective RANK receptor in pre-osteoclasts (Fuller et al., 1998).

The interaction RANKL-RANK allows the differentiation and maturation of pre-osteoclasts, which have and hematopoietic (bone marrow) origin (Walker, 1973)

Osteocytes register the stimuli of activation, and it can be hormonal; changes in the bone structure are caused by damage of aging and external fields.

3) Resorption phase

Bone resorption occurs when the osteoblasts, located on the bone surface, decompose the osteoid matrix and release calcium ions into the bloodstream (Rowe P, Koller A, 2021). It is produced by various proteolytic enzymes such as matrix metalloproteinases (MMPs), gelatinase, and collagenase (Meikle et al., 1992).

The osteoid matrix is an unmineralized and organic matrix before the maturation of the bone tissue. In mineralization, the matrix becomes acid (pH=4) due to the pumping of H^+ ions into the bone, allowing organic components such as collagen and others to be

degraded by osteoclasts' proteins secreted. Once the process is finished, osteoclasts die by apoptosis (Manolagas, 2000).

The end of the resorption phase and the beginning of the formation phase occur through a coupling mechanism in the resorption lacunae, where it is ensured that the amount of matrix that is formed is equivalent to that previously reabsorbed (Parfitt, 1982).

It has not yet been determined whether osteoblast activation begins simultaneously with osteoclast recruitment or later during lacunar development. However, it is presumed that during resorption, the osteoclasts release local factors that inhibit their function (negative regulatory effect) and, on the other hand, stimulate the activity of the osteoblasts (positive regulatory effect) (Hill, 1998); the process is known as "coupling." A scheme is visualized in Figure 9.

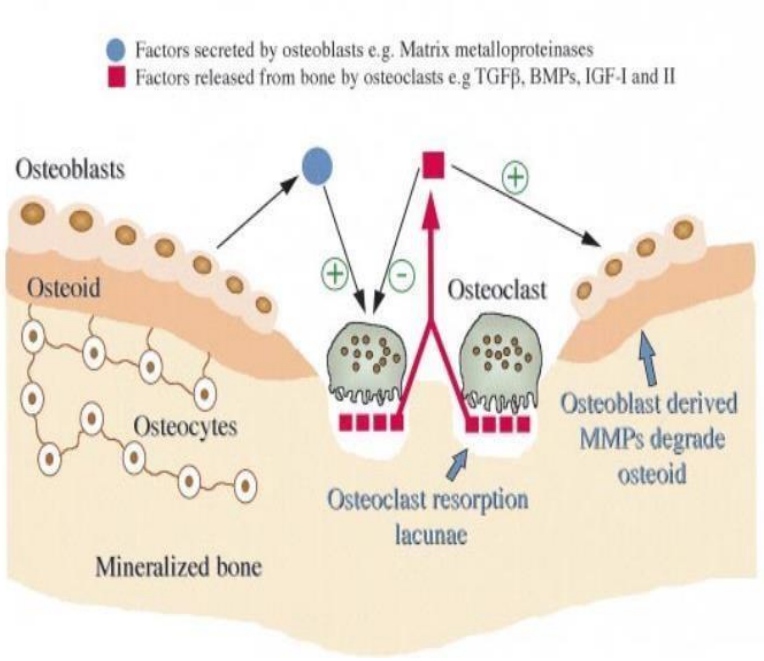

Figure 9. Diagrammatic representation of the coupling process. The osteoclastic bone resorption is followed by osteoblastic bone formation. First, the synthesis and release of matrix metalloproteinases (MMPs) by osteoblasts expose the mineralized matrix. Then, the osteoblasts stimulate osteoclast activity and inhibit their activity due to TGF-β and BMPs factors. By (Hill, 1998)

When osteoclasts complete the resorption cycle, they secrete proteins that serve as a substrate for osteoblast attachment (Canalis, 1983) and die by apoptosis, characterized by nuclear and cytoplasmic condensation and DNA fragmentation. The growth factor, TGF-β, blocks bone resorption and can induce apoptosis, while osteoclast stimulatory factors such as parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D3 inhibit apoptosis (Roodman, 1996).

4) Formation phase

After the resorption phase, a reversal phase begins and precedes the formation phase. The reversal phase lasts ~9 days (Hill, 1998). It is characterized by the absence of osteoclastic activity due to the short half-life of osteoclasts (~ 12,5 days), the progressive accumulation of calcium in the resorption lacunae that directly controls the osteoclastic activity, and, in the long term, inhibiting it (Zaidi, 1990) and the osteoclasts inactivation and osteoblasts attraction by TGF-β (Mundy, 1991).

Osteoclasts will disappear during the reversal phase, and macrophage-like cells will appear on the bone surface to remove matrix residues thanks to their high collagenase concentration.

The formation phase results from complex biochemical cascades with a proliferation of mesenchymal cells, maturation of pre-osteoblasts, matrix formation, and mineralization.

The pre-osteoblasts synthesize a substance where the new tissue will adhere and express BMP proteins that help in cell differentiation (Gómez, 2008). Afterward, the osteoblasts gather in the resorption lacuna and form the osteoid that will take ~13 days to mineralize at an initial rate of 1 µm/day (Eriksen et al., 1986). The osteoblasts will continue their work until the lacuna is filled.

Figure 10 summarizes the stages of the remodeling cycle.

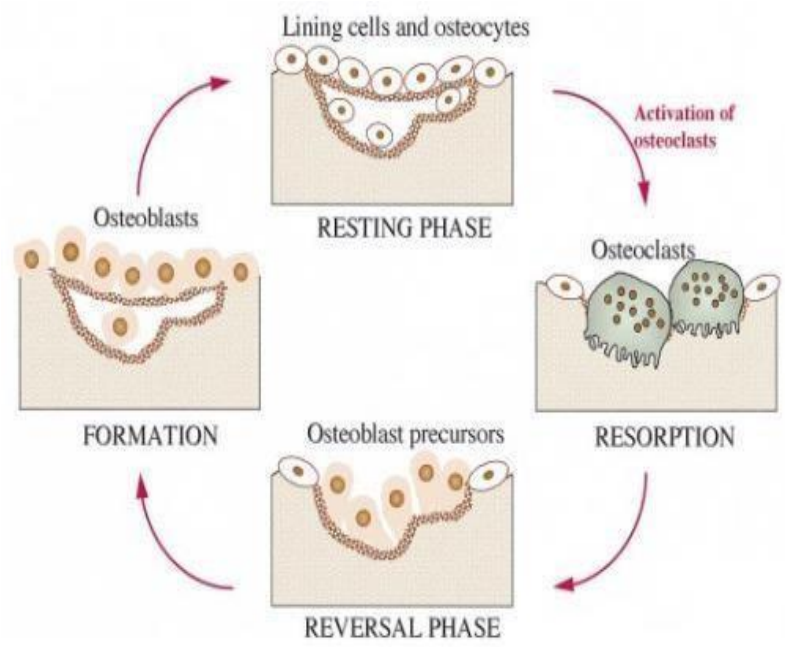

Figure 10. Stages of bone remodeling. During the resting phase, multinucleated osteoclasts derived from bone marrow monocytes are activated and resorb a discrete area of mineralized bone matrix. During the reversal phase, the pre-osteoblasts cells migrate into the resorption lacuna and disclose the former osteoclastic activity. During the formative phase, the osteoblasts form a new unmineralized bone matrix called osteoid. Finally, the osteoblasts are embedded in osteoid lying on the newly formed bone's surface, and the cycle begins again. By (Hill, 1998)

5) Mineralization phase

Biomineralization occurs when minerals are deposited either inside or on the organism's tissues (Boskey, 1998). In bone tissue, mineralization consists of the deposition of hydroxyapatite crystals at regular and parallel intervals on collagen fibrils.

Mineralization occurs in two phases. The first begins with hydroxyapatite crystals within matrix vesicles, and the second is the propagation of hydroxyapatite within the extracellular matrix (Negri, 2011). The matrix vesicles are small vesicles on the surface of osteoblasts, chondrocytes, and odontoblasts. It has a diameter of 50 to 200 nm and it is

rich in annexins A2 (II), A5 (V), A6 (VI), and various phospholipids, among which are alkaline phosphatase, nucleotide pyrophosphatase/ phosphodiesterase 1 (NPP1), and especially phosphatidylserine which has a high affinity for calcium (H. C. Anderson, 1995; Wuthier, 1975), in addition to the cotransporter Na/Pi III and $PHOSPHO_1$.

5.1) The First phase

The phospholipids and calcium-binding proteins present within matrix vesicles, such as phosphatidylserine, calbindin D9K and bone sialoprotein, promote calcium accumulation (H. Anderson, 2007; H. C. Anderson, 1995).

Annexins form channels that incorporate calcium within vesicles. The Na/Pi type III cotransporter and the cytosolic phosphatase $PHOPHO_1$ provide inorganic phosphate (Pi). The hydroxyapatite is formed in the vesicles when calcium and phosphorus accumulation is greater than the calcium phosphate's solubility point ($CaPO_4$) (Negri, 2011).

5.2) The Second phase

The hydroxyapatite formed in the first stage penetrates the matrix vesicle's membrane wall and elongates within the extracellular matrix space. For the continuous formation of new hydroxyapatite crystals, the extracellular fluid must contain enough calcium and inorganic phosphorus (Pi). Finally, the hydroxyapatite crystals around matrix vesicles fill the spaces between collagen fibrils (Ali et al., 1970; H. C. Anderson, 1969).

Once the bone matrix has been entirely mineralized, inorganic pyrophosphate (PPi) inhibits hydroxyapatite crystals' formation (Negri, 2011). The crystal propagation depends on the ratio of inorganic phosphate (Pi) to inorganic pyrophosphate (PPi).

The process is completed at approximately 90 days in the trabecular bone and 130 days in the cortical bone (Gómez, 2008) until the remodeling site is filled and the cycle is completed. Once the cycle is finished, the bone remodeling is repeated.

Regulation of bone remodeling

Bone remodeling is a complex process where multiple hormones and growth factors work together. During the human being's life, the bone undergoes formation and destruction to deliver calcium to the blood (Muzzo, 2010).

Parathyroid hormone (PTH), vitamin D, and calcitonin help maintain normal calcium and phosphorus levels, essential for mineralization, acting on the intestine, bones, and kidneys. At the bone microenvironment, local factors (Table 2) are responsible for regulating, formation and resorption. The TGF-β activates osteoblasts and inhibits bone resorption. Also, the IGFs, FGF, and PDGF affect osteoblast proliferation and differentiation (Hill, 1998).

Hormones and growth factors are responsible for directing the bone remodeling process.

Factors that affect bone remodeling

Calcium absorption decreases with age and calcium intake, affecting bone mineralization. Likewise, drugs such as antacids, anticonvulsants, anticoagulants, diuretics, and glucocorticoids negatively affect the calcium balance. Other disturbing factors are excessive consumption of alcohol, coffee, and tobacco.

Bone mineral density (BMD) is a test that measures the number of minerals contained in a volume of bone (Shriver, 2020). A low result can favor osteoporosis and can occur in patients with a low calcium intake or chronic diseases and patients with a lack of physical activity or who have had prolonged bone immobilization.

2.4.5. Bone healing

The process where new tissues replace injured tissues with the same shape and characteristics of healthy tissue is regeneration. Bone tissue consists of a highly vascularized characteristic that favors the regeneration or bone healing capacity where osteogenesis is promoted. This process is carried out from when the bone is fractured into two or more pieces until it is welded again (Shi & Kou, 2018).

The initial inflammatory phase was beginning when the injury occurred. The fracture tears the fracture's blood vessels and adjacent white tissues, causing blood accumulation (Gómez, 2008). Fibrin, a protein present in the blood, is a crucial component for bone repair as it allows the formation of a hematoma.

The damaged area presents hypoxia due to the blood vessels' breakdown; consequently, the pH decreases, the acute inflammation begins immediately, causing edema, pain, and inflammation in the damaged area and adjacent areas. Subsequently, the tissue located at the fracture edges is reabsorbed from 1 to 2 mm; this reabsorption causes
the fracture lines to be seen on an X-ray five to ten days after the injury (Sánchez & Salerni, 2015). The proteolytic degradation of the extracellular matrix provides activation factors for monocytes and macrophages, and once these are activated, they release fibroblast growth factor (FGF) that stimulates the production of pro collagenase plasminogen (Gómez, 2008).

Proliferation occurs during the vasoconstriction to avoid contaminants or infectious agents' arrival and occurs vasodilation to allow platelets and other actors to arrive at the consolidation. Blood forms a clot in the area of injury, and platelets release modulating factors (I PDGF, TGF-β, and FGF) and promote hemostasis (Grimaldo-Gómez, 2017).

The repairing phase develops once the proliferation phase has ended. Here, the blood clot will begin to disappear, and new blood vessels will develop from outside the bone to supply nutrients as a soft callus. The fibrous inner and outer layers of the periosteum, the endosteum, and the medullary cells participate in callus formation. Collagen will be where cells will anchor, differentiating into osteoblasts and chondroblasts. The soft callus forms like a collar of endochondral bone around the fractured area, and it has cartilaginous consistency and hardens progressively forming the hard callus, as endochondral mineralization and calcification occurs during the remodeling phase (Sánchez & Salerni, 2015).

The last phase is remodeling, a previously described activation-reabsorption-formation process where the endochondral callus completely ossifies, and the bone undergoes structural remodeling (Gómez, 2008).

Bone repair involves biological and mechanical processes; sometimes, some fractures heal slowly or are not repaired despite optimal treatment. It is difficult to provide an approximate time frame for each phase, as the cure rate varies according to each patient's age and characteristics. Factors such as age, bone type, lifestyle, and diet influence repair time and effectiveness, so it is necessary to use alternative or complementary methods. Below, some non-invasive technologies used to impact bone repair.

Orthopedic immobilization with casts or continuous extension achieves that the fractures heal without a problem; however, orthopedic treatment requires prolonged immobilization of the adjacent muscles and joints, leading to muscle atrophy and a significant risk of joint stiffness. The callus must be subjected to the progressive increase in stress through the progressive de-stiffening of the fixing medium, which can be internal or external, and in this way, the risk of recurrent fracture is minimized (J.-P. Meyrueis &

Cazenave, 2004). It is presumed that the mechanical forces act by producing an electrical signal that induces osteoinductive factors.

Regarding stimuli based on electrostimulation, they are based on the fact that the electric fields that are produced in the bone environment can modulate cellular activities, which is the reason it seeks to develop various systems for electrostimulation for therapeutic use based on the same principle (I. S. Kim et al., 2006, 2009). Electrostimulation equipment can be direct current, capacitive coupling and inductive coupling.

Various studies have suggested that electromagnetic stimulation has a direct impact on the growth factor cascades (Roy K. Aaron et al., 2004; Guerkov et al., 2001; Lohmann et al., 2003), regulation of collagen (Ciombor & Aaron, 2005), production of cytokines (Spadaro, 1997) and proteoglycans. These pathways allow the Calcium-Calmodulin signaling cascade's stimulation favoring bone healing (Haddad et al., 2007; F. R. T. Nelson et al., 2003).

In 1970, magnetotherapy was presented as a treatment for fractures consisting of the induction of electrical waves in the bone generated by an electromagnetic pulse to improve its healing with biostimulant, anti-inflammatory, and edema reduction effects (Ángel et al., 2002). Also, it exerts piezoelectric bone effects and collagen since the crystals are subjected to mechanical stress, which induces the consolidation of the bone, accelerating its recovery.

2.4.6. Bone piezoelectricity

In 1880, the French physicists Jacques and Pierre Curie discovered a phenomenon in individual crystals in response to applied mechanical stress (Katzir, 2006).

The piezoelectric effect results from a mechanical tension, which causes the material to acquire an electrical polarization; in turn, a potential difference and electrical charges appear on its surface (Nimrod, 2019), as shown in Figure 11. The piezoelectricity is a reversible process; when crystals stop receiving an external voltage, they regain their shape.

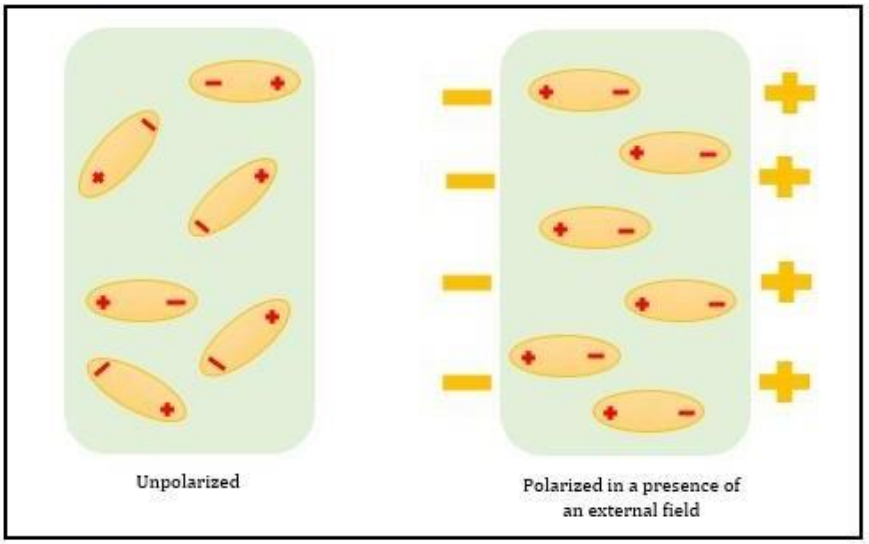

Figure 11. Electric polarization

Direct Piezoelectricity: The ability of a material to produce voltage under tension or compression.

Indirect Piezoelectricity: The bending caused in piezoelectric materials due to applied potential or electric field.

The first scientific observation of the piezoelectric effect in biological tissues dates back to 1941, after observing electrical polarization in hair and wool (Martin, 1941). Eiichi Fukada and Iwao Yasuda were scientists who laid the groundwork for bone piezoelectricity. In 1956, after small subject bone and collagen plates to different stress types, they established the mechanism of induced stress and concluded piezoelectricity also exists in bone and related tissues (Fukada, 1956).

The piezoelectric effect in bone has been suggested as the cause for Wolff's law: *"The form of a bone being given, the bone elements place or displace themselves in the direction of functional forces and increase or decrease their mass to reflect the amount of the functional forces"* (Wolff, 1892).

The density, shape, and size of a person's bones depend on the magnitude and direction of the mechanical loads acting on the bones. In accordance, bone remodeling may be influenced by electrical dipoles produced by the piezoelectric effect due to bone microstructure orientation of collagen anisotropy when tissue is subjected to mechanical stress.

There are divided studies about the influence of hydroxyapatite crystals. So far, it is a fact that both are crystalline structures, anisotropic and lacking a center of symmetry. Part of the limited knowledge about hydroxyapatite's behavior is due to the nano-scale (~ 50 x ~ 25 x 2 nm) of bone crystals, so the properties are studied in long crystals and the results are assumed for the bone hydroxyapatite (Pawlikowski, 2017).

During mechanical stress, the HA plates bend, which generates electric potentials in the crystals that compose it. When walking, the pressure is exerted on the lower extremities, and therefore, on the crystals of the inorganic matrix; this undoubtedly causes the creation of currents of direction up and down and vice versa (Pawlikowski, 2017).

Hydroxyapatite is a hexagonal structure (Figure 12) arranged as a pile of phosphate groups ($PO4^{3-}$) with two tunnel-shaped regions parallel to its *c* axis. One of the tunnel-shaped regions is occupied by OH^- ions while Ca^{2+} ions occupy the other (Escobar, 2018).

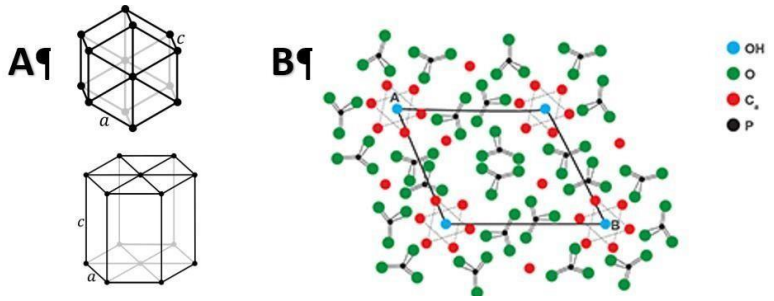

Figure 12. Crystal structure of HA. (A) The hexagonal crystal system of HA: In general, a hexagonal crystal system with three axes of equal length is located in the same plane and intersecting at 120 ° angles. The fourth axis is longer or shorter than the others and is perpendicular to those already mentioned. (B) Chemical structure of hydroxyapatite. By (Escobar, 2018)

Hitmi et al. (1986) propose that the piezoelectricity of HA originates from the reorientation of dipoles in the tunnels occupied by OH^- groups. On the contrary, Minary-Jolandan & Yu (2009) and Vannessa & Cerrolaza (2013) propose that HA is a center-symmetric crystal; therefore, it is excluded that it is piezoelectric, taking as a model the study carried out by Marino et al. (1971) where they took samples of human bone to measure the piezoelectric effect after demineralization or decollagenation of the samples; the results indicated that a large part or possibly all of the piezoelectric effect found in the whole bone comes from the organic component. In addition, the hydroxyapatite present in animal bone tissue is non-stoichiometric and non-centrosymmetric; however, it has been found that it shows piezoelectric properties.

When collagen molecules (which are load carriers) submit to tension, the internal charges move to the surface producing an electric potential. The electric potential causes tension that produces an indirect piezoelectric effect in bone; moreover, this effect is detected by the osteocytes in the areas of great stress (C. A. L. Bassett & Becker, 1962). Subsequently, the osteoblasts deposit minerals (calcium primarily) in the tension place, starting remineralization, increasing bone mineral density (BMD) (Vannessa &

Cerrolaza, 2013). Stresses such as tension or compression generate modifications in multiple oriented collagen fibers to evoke physiologically substantive and orientation specific changes.

Although Marino et al. (1971) assure that the inorganic matrix by itself does not contribute to the piezoelectric effect, subsequent investigations yielded new perspectives that are included below.

Pawlikowski (2017) reported that the electrical currents generated during stress promote the bonds between collagen and hydroxyapatite, so the absence of these can weaken the same even breaking, resulting in weakening the bone structure. Likewise, changes in external atmospheric pressure and gravity changes can generate a current that also affects the bonding bonds. The lack of gravity increases bone and muscle wear, which can be seen in astronauts' bone structures who have been in space for a specific time.

The influence of the hydroxyapatite-collagen interaction is also observed in that the HA crystals cover the collagen fibers and prevent an excess of water within them, thus avoiding a possible swelling of the matrix (Ahn & Grodzinsky, 2009; J. C. Anderson & Eriksson, 1970). Studies hold that even in a 100% relative humidity environment, collagen in bone has only about 12% moisture content (Reinish & Nowick, 1975).

The collagen fibers' gaps are filled with hydroxyapatite and water (Weiner & Wagner, 1998), the latter being of 3 types: free, bonded, and structural. On the one hand, the free and bonded water acts directly on the collagen strand's surface, forming an interconnected network. On the other hand, the structural water is responsible for maintaining the collagen strand's integrity by forming a bridge between the alpha chains strengthening thestructure. The water present allows the collagen to remain moist, allowing the correct

passage of generated currents. In wet environments, piezoelectricity increases at the fibers' ends (Sulca Buitrón, 2019; Vallet-Regí & González-Calbet, 2004; Weiner & Wagner, 1998).

3. Effects of external fields on collagen piezoelectricity and bone tissue remineralization

Once all the processes to which the bone is subjected during life and the influence of piezoelectricity for their correct development are known, then a literary review about the effects of the mechanical, electrical, and magnetic fields in collagen piezoelectricity and bone tissue remineralization is presented.

3.1 Mechanical force fields: Types, quantities, and application methods

Throughout life, bone is exposed to high-magnitude mechanical stimuli derived from daily activity, generating small deformations (0,1% deformation); also, the skeleton is continuously subjected to very low-magnitude stimuli (deformations <0,0005%) and high frequency (10-50 Hz), product of the constant muscular contractions necessary to maintain posture (Delgado-Calle & Riancho, 2013; Huang et al., 1999). The types of loads to which bones are subjected are shown in Figure 13, although most bones experience a combination of all loads.

Strength is derived from applied forces that can be internal (muscle movement) or external (an applied load). Pearson & Lieberman (2004) defined as essential parameters stress (σ, force, F, per unit area, A) and strain (ε, the change in length, ΔL, per unit length, L).

According to the International System (SI), stress is expressed in newtons per square meter (1 N/m^2 = 1 Pa), or in N/mm^2 (1 N/mm^2 = 1 megapascal or MPa) (P. Meyrueis et al., 2004), while a strain is a measure of shear expressed as micro-strain (με), or as a percentage (%) of change in dimension (Hart et al., 2017). The interaction of stress and strain provides information about bone's mechanical behavior when it deforms under load.

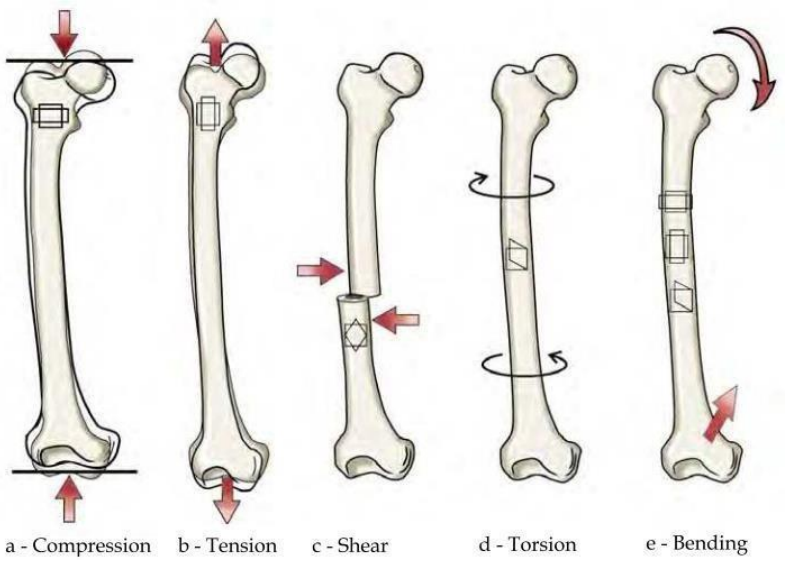

Figure 13. Loads subject to the bone. (A) Compression tends to shorten and widen. (B) Tension tends to stretch and narrow. Both act perpendicular to the surface. (C) Shear acts surface's parallel. (D) Torsion causes perpendicular stresses to the neutral bone axis (E) Bending includes all changes seen in A, B, and C. By (Dalla & Bankoff, 2012)

The elasticity and tension resistance abilities of bone tissue are due to the cross-linking of collagen molecules; in contrast, the stiffness and compression resistance are due to the mineral component. Both in combination create an overly strong and strength tissue in response to applied forces (Akkus et al., 2004; Pearson & Lieberman, 2004; Zioupos, 2001).

One of the action mode hypotheses is that, as the bone deforms, the osteocytes act as mechanosensors and osteoblasts act as stress sensors. As a consequence of the deformation, intracellular fluid production increases, stimulating cellular communication and generating small electrical charges (Cowin et al., 1995).

A complete hypothesis is a *Streaming-Generated Potential (SGP)* attributed to piezoelectricity and streaming potential. When a mechanical load is applied as a function of time, the intracellular fluid flow induces a stress-generated flow potential;consequently, the deformation increases the levels of PKC (protein kinase C) and Ca^{2+} flux in osteoblasts and osteocytes, in turn, stimulate the release of PGE2 (prostaglandin)

and nitric acid (NO), that are potent bone growth regulators. The electrical polarization changes the aggregation of macromolecules and ions in the extracellular matrix and stimulates cells that remodel bone architecture until the signal is no longer produce (Rajabi et al., 2015).

The bone adapts to mechanical loads through the Harversian modeling, as shown in Figure 14. The Harversian modeling consists of modeling and resorption process and is influenced by mechanical loading. Excessive loading can damage the tissue, which stimulates remodeling, while lack of loading or disuse causes a slowdown in bone formation and, therefore, rapid resorption of bone mass. (Burr et al., 1997).

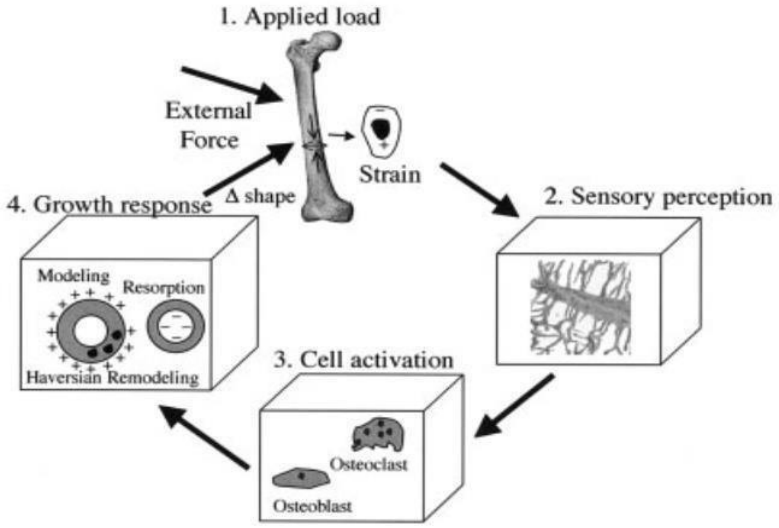

Figure 14. Response of bone to loading. By (Pearson & Lieberman, 2004)

The bone's adaptive response involves aspects such as type of magnitude, range, frequency, distribution of strain, the number of load cycles, and rest periods. Cortical bone is stiffer than trabecular bone, hence can tolerate higher stress (~150 MPa) but lower strain (~2% yield) before failure; in contrast, trabecular bone has better elasticity than cortical bone due to its porous nature, thus tolerate lower levels of stress (~50 MPa) but much higher strain (~50% yield) before failure (Hart et al., 2017) as shown in Figure 15.

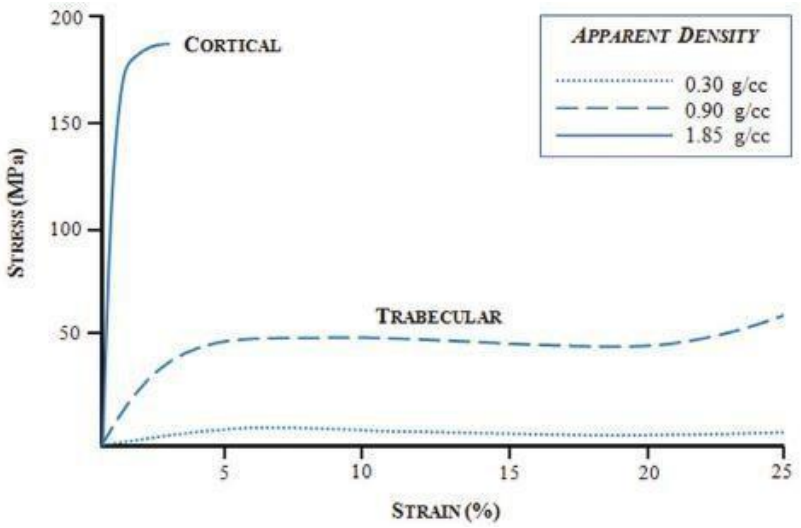

Figure 15. Stress-strain curve. Cortical bone is stiffer with a low resistance to strain and high-stress resistance. In contrast, trabecular bone is porous with a low resistance to stress and high resistance to strain. Taken from (Hart et al., 2017)

With this present knowledge, several experiments have been developed *in vitro* and *in vivo* to understand the influence of mechanical loading on collagen's piezoelectricity and bone tissue remineralization.

The percentage of deformation differs between experimentation techniques since the tension necessary to produce osteogenesis is higher (1-10%) *in vitro* than *in vivo* experiments (0,04- 0,3%), possibly to the amplification of the extracellular fluid produced by the tension (You et al., 2001). Resulting in a disadvantage when testing hypotheses because although *in vitro* studies can provide critical experimental results, these can only be validated when performed *in vivo* (Pearson & Lieberman, 2004).

The extracellular fluid flow seems to be one of the critical pieces for the bone tissue's piezoelectricity; as the loading rate increases, the flow pressure will also cause a more significant cellular response (Turner, 2007). Dynamic loading allows the extracellular fluid flow pressure to continually change as tissue deformation changes in response to mechanical loading; controlled *in vivo* experiments completed by Hert during 1969 and

1972 concluded that osteoblasts respond only to dynamic loading, not static load (Hert et al., 1969, 1972). Similar experiments in different animal models were subsequently replicated, as seen in Figure 16.

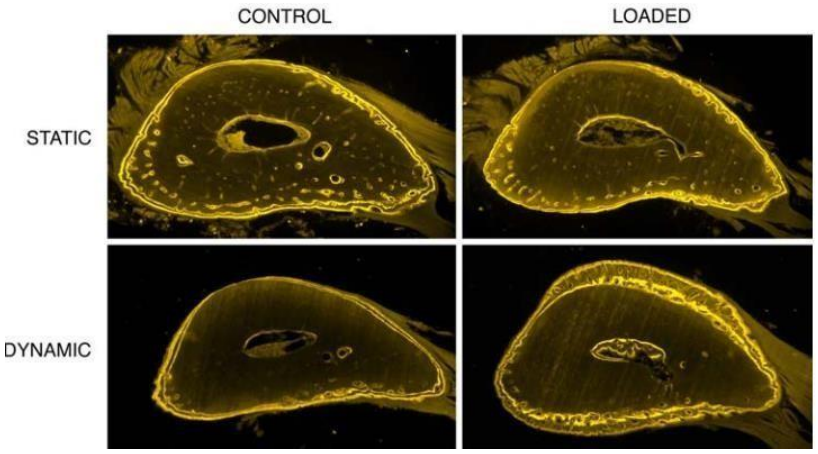

Figure 16. Section from the rat ulna subjected to a static and dynamic load of 2 Hz. The bright lines along the surface of the periosteum show mineralization. Static loading minimally inhibits tissue formation in the periosteum, while dynamic loading significantly improves tissue formation.

Taken from (You et al., 2001)

Mechanical loading also influences collagen alignment. Cortical bone tissue located in regions where the predominant tensions are tensile has a higher percentage of collagen fibers aligned along the bone axis. In contrast, regions where the predominant tensions are compression, have a higher percentage of collagen fibers aligned transversely along the axis (see Figure 17) (Riggs et al., 1993). This observation is essential, as applying mechanical stresses over a long period allows the collagen fibers' predominant orientation to change as the tissue remodels, suggesting that locally applied mechanical stresses affect collagen formation (Turner, 2007).

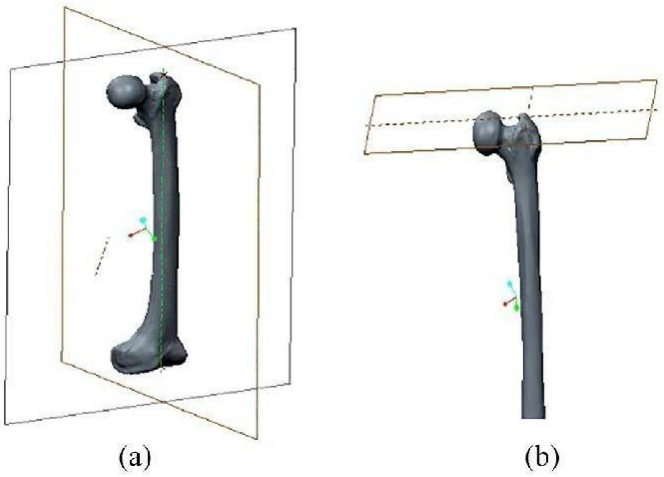

Figure 17. a) Longitudinal axis b) Transversal axis. By (Uddin et al., 2019)

Different studies document that bone mass increases at sites directly affected by the impacts of specific muscle forces or contractions (Heinonen et al., 2002; Nordström et al., 1998; Slemenda et al., 1991; Söderman et al., 2000). The right ulnae of 26 adult female rats were subjected to 360 load cycles/day for 16 weeks, and observations were made concluding that on the surfaces where the stress was more significant (distal mid-shaft), bone formation occurred, unlike the surfaces where the stress was lower (middle distal diaphysis) were no bone formation occurred (Robling et al., 2002, 2006), as shown in Figure 18.

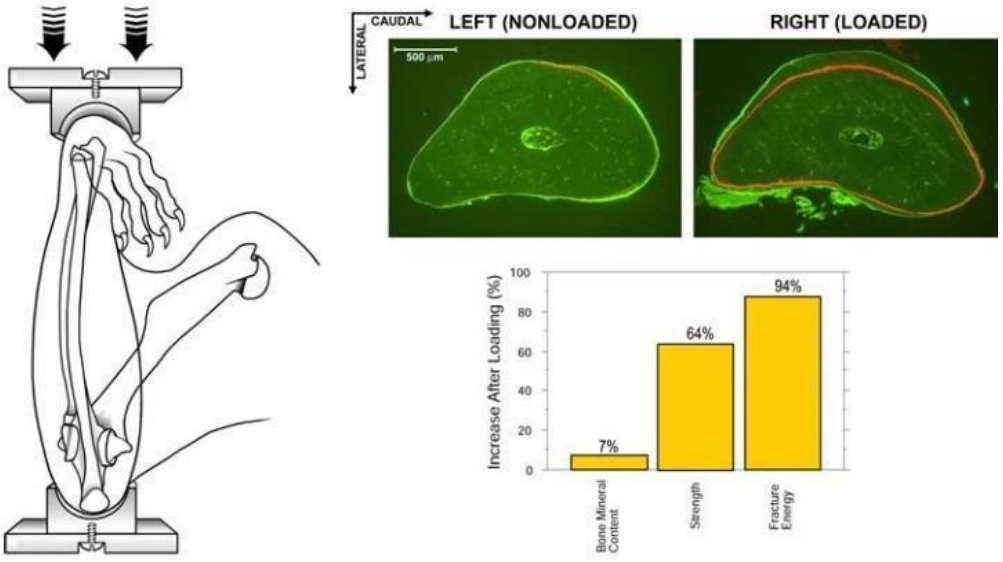

Figure 18. To measure the effect of mechanical stress on a rat ulna, an axial load was applied to the upper extremity causing the bone to bend so that the medial surface is under compression and the lateral surface is under tension (panel left). The bone formation is evident in the sectional cut (top right); the red lines indicate the tissue's edge at the beginning of the experiment; after loading, the bone tissue protrudes from the red lines. Bone strength increased by 64%, and the energy required for the fracture increased by 94%, while the mineral content increased by only 7%. Taken from (Robling et al., 2002)

The deformation frequency represents the number of cycles applied per second to a given structure (Hart et al., 2017). Experimental studies show that long bones need to be loaded at 0,5 Hz or more to initiate tissue formation (Robling et al., 2002). As the loading frequency increases, the required mechanical loading decreases, it is suggested that a loading frequency of 30 Hz, the bone stress would be only 65 με, enough to initiate new tissue formation (Turner, 2007). Therefore, increasing the loading frequency to initiate bone formation would improve the methods of applying mechanical loads.

Bone responds non-linearly to strain frequency, and osteogenic adaptations cease to intensify beyond a 10 Hz stimulus cycle due to signal saturation (Hsieh & Turner, 2001; Judex et al., 2007; Reis et al., 2011; Warden & Turner, 2004). Experiments carried out (Burr et al., 2002; Umemura et al., 1997; Wu et al., 2009) probe that ~ 95% of mechanosensitivity is dampened after ~ 20 to 40 loading cycles in physiological strain

magnitudes (~ 2000 με in compression), with almost no discernible osteogenic benefit established beyond ~ 100 loading cycles.

Now classic experiments have shown that bone cells quickly saturate and lose mechanosensitivity. The *in vivo* experiment carried out by Rubin & Lanyon (1984) in turkeys showed that only 36 cycles/day at physiological strain magnitudes were as effective in promoting bone formation as 1800 cycles/day at the same strain magnitude. Moreover, Umemura et al. (1997) trained rats to jump multiple times a day, resulting in increasing bone mass in the tibia and femoris. However, the anabolic response was saturated around 40 loading cycles. As a result, the osteogenic response can be enhanced by regimens that incorporate short, vigorous bouts of dynamic loading followed by rest periods.

Figure 19 shows the data plotted together, the resulting graph from that the relationship between the number of cycles and tibial bone mass in the Umemura study is identical to that between the number of cycles and ulnar bone mineral content the Rubin & Lanyon study. There was an approach to saturation in both cases as the duration of loading was increased from 36 to 720 cycles/day. So, increasing the duration of a loading bout results in diminishing returns in bone formation (Burr et al., 2002; Hsieh & Turner, 2001).

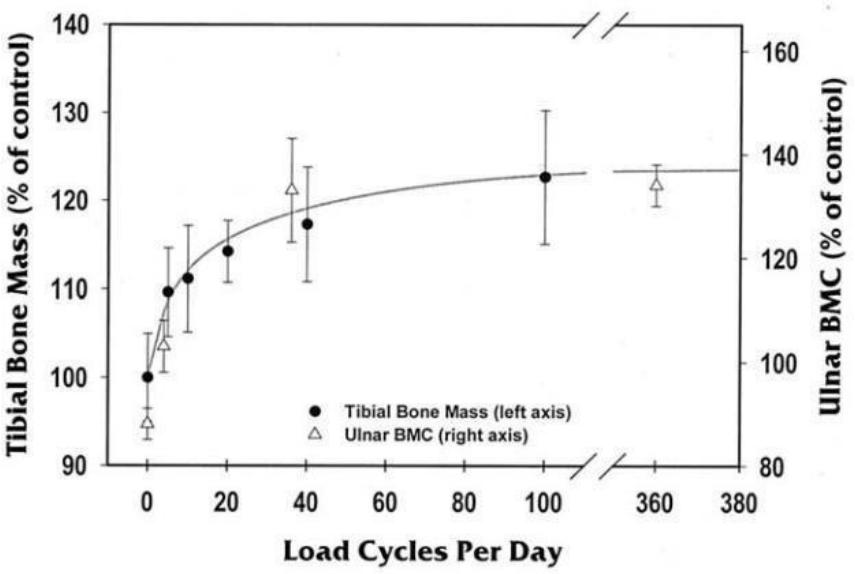

Figure 19. Bone mass of rats (•) and turkeys (Δ). The mechanical loading saturates as the number of loading cycles increases. There is a limited benefit of additional loading cycles above about 40 cycles per day. Taken from (D. Burr et al., 2002)

In conclusion, throughout life, the bone experiences different loads, whether due to growth or physical activity, so it is necessary to develop mechanisms to support the mechanical load. The composition of the extracellular matrix plays an essential role since the combination of elasticity and resistance creates a muscular tissue that is rigid but at the same time plastic.

Likewise, it is known that osteoblasts initiate the formation of new tissue in areas where stress occurs. Although the detailed mechanism is still not fully understood, the *Streaming-Generated Potential (SGP)* theory proposes a series of actions where different bone molecular components are involved, such as intracellular fluid, growth factors, and cells.

The disadvantage of applying external mechanical fields resides in the difference between the percentage of deformation in the in vitro and in vivo studies, which hinders its development and experimentation in the laboratory, delaying the new findings. Also,

osteoblasts lose mechanosensitive after a defined period, so it is essential to include periods of rest between periods of loads, which can also prolong new tissue growth.

The benefits of the application of external mechanical fields are the controlled application of load and its ease of use since it is a non-invasive technique; also, until now, it is impossible to stimulate the bone in an isolated way, so the muscles are also stimulated skeletal that also increase their mass. It is essential to take into account that the formation mechanisms are activated after a defined load magnitude. If the load is greater than the threshold, the bone fractures and the repair mechanisms are activated, while if the load is less than the activation, the resorption mechanisms are activated.

3.2 Electric fields: Magnitudes, frequencies, and voltages used, application methods

The use of electrical stimulation for osteogenesis is a technique dating from the '60s; Fukada & Yasuda (1964) found that the fractured bones have electrical currents which activate endogenous bone repair mechanisms. Following this principle, the use of external electrical stimulation was proposed to activate the repair mechanisms, concluding that an external electrical field's application causes a direct piezoelectric effect in the bone.

Generally, the electric current is the load flow that traverses a material due to electrons' movement within it. According to the International System (SI), the current is expressed in coulombs per second (C/s) = ampere (A). Since a movement of charges produces the electric current, it generates a magnetic field (Serway et al., 2004).

Depending on the type of electrical flow, the current can be direct (DC) or alternating (AC). Figure 20 shows a representation of the electron movement of each one.

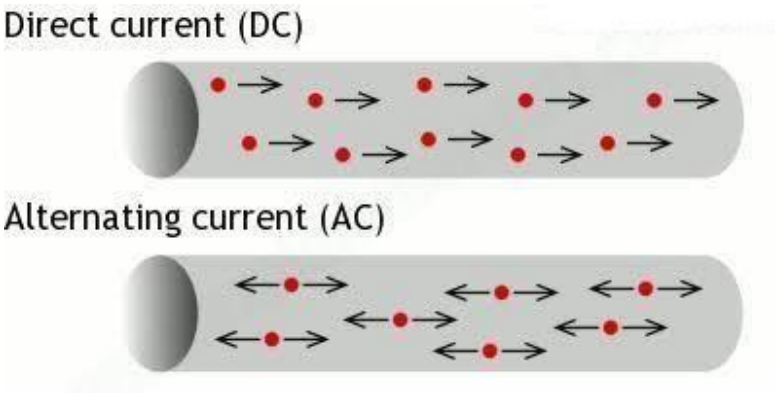

Figure 20. Direct current (DC) and alternating current (AC). ***Direct current (DC)*** *is characterized by not varying in time, magnitude, or direction. It circulates through a closed circuit where the electrons move from the negative pole to the positive pole, and it can be direct current, pulsating direct current, or alternating current. On the other hand,* ***alternating current (AC)*** *is characterized by the fact that the electrical flow varies in magnitude and direction, alternating in periods. By* (Serway et al., 2004)

The electric field stimulation's effectiveness relates to the direct current improving the consumption of dissolved oxygen and increasing the pH in the cathode vicinity, stimulating chondroblasts, osteoblasts, and chondrocytes to increase significantly in the areas of healing. Studies have shown that when applying direct current stimulation that varies between 5 and 100 µA of different polarities, the negative direct current of 20 µA is the optimal one to provoke osteogenesis. Lower currents produce minimal stimulation, but without bone growth, and currents are more significant than 390 µA cause bone demineralization and even necrosis, as does positive current (Guzelsu & Demiray, 1979; Andrew A Marino & Becker, 1970; Norton & Moore, 1972; Vannessa & Cerrolaza, 2013). The ES (electrical stimulation) methods are applied direct current (ADC), capacitive coupling (CC), and inductive coupling (IC) (Sulca Buitrón, 2019).

Gan et al. (2005) define ADC's stimulation as an invasive method that consists of cathodes connected to a power source serving as an anode. The device is surgically implanted with the cathode placed at the bone defect and the anode directly to the soft tissue. The power supply provides a constant current between 5 and 100 µA through electrodes that can vary from 2 to 4 units. Once healing is complete, the device is removed.

Likewise, capacitive coupling (CC) stimulation is a non-invasive method. The device consists of two electrodes connected to the AC signals generator; the electrodes are placed on the skin on opposite sides to the required site of stimulation, so requires the use of a conductive gel Gan et al. (2005). The use of potentials from 1 to 10 V and frequencies between 20 to 200 kHz that produce an electric field whose magnitude ranges from 1-100 mV/cm, effective for bone stimulation (X. L. Griffin et al., 2011)

The third method of producing electrical stimulation is inductive coupling (IC). It is a non-invasive method, where one or two carrier current coils are placed on the skin over

the fracture site (Evans et al., 2001) and exposed the fractured bone to the influence of a pulsed electromagnetic field therapy (PEMF). The electric field that forms varies in size due to the tissue nearby at the fracture site and the applied magnetic field properties (Roy K Aaron et al., 2004). The electromagnetic fields used vary from 0,1 to 20 G and create an electric field at the fracture site of 100 mV/cm (R K Aaron & Steinberg, 1991).

Figure 21 shows a diagram of the three administration methods of electrical stimulation.

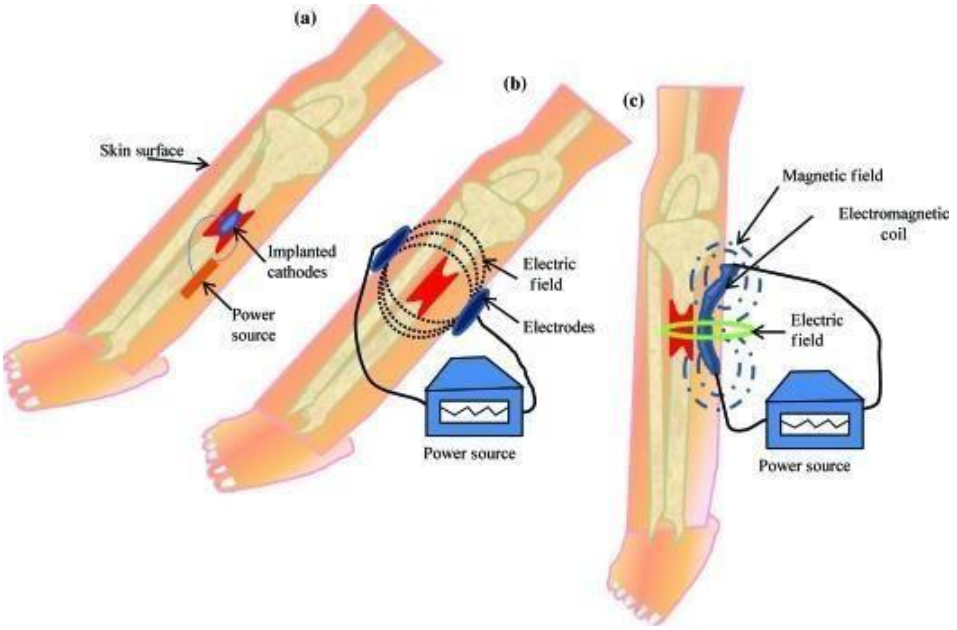

Figure 21. The three administration methods of electrical stimulation. ***(a) Direct current (DC):*** *The cathode is implanted at the fracture site and is connected to a source of energy, either subcutaneous or external, to generate an electric field at the fracture site.* ***(b) Capacitive Coupling (CC):*** *Capacitive coupled electrodes are placed on the skin side to side of the fracture site and connected to an external power source that induces an electric field at the fracture site.* ***(c) Inductive Coupling (IC):*** *An electromagnetic current-carrying coil is placed on the skin overlying the fracture site and connected to an external power source. The coil generates a magnetic field that causes an electric field at the fracture site.* By (M. Griffin & Bayat, 2011)

In vitro and *in vivo* studies appear to understand the influence of external electric fields on collagen piezoelectricity and bone tissue remineralization, with and without the presence of bone cells.

To understand the effect of external electrical stimulation on the growth factors cascades, Gan et al. (2005) carried out an experimental study in rabbits treated with ADC, CC stimulation, and passive devices (control). The power supply delivered a constant current of 20, 50, or 60 μA through the cathodes to the fusion site. The results showed that the DC-stimulated group's tissue increased the $_m$RNA expression levels of the bone morphogenetic proteins BMP-2,6,7, and the ALK2 (Activin receptor-like kinase-2) were higher than the control group. In contrast, the group stimulated with CC had higher levels of $_m$RNA expression of BMP-2,4,6,7, TGF-β1, FGF-2, and VEFG than the control group. CC also promotes transmembrane calcium translocation through voltage-gated calcium channels, subsequent activation of calmodulin, and a prostaglandin increment.

Furthermore, DC stimulation allows the electrochemical reaction $O_2 + 2H_2O + 4e^- \rightarrow 4\ OH^-$ to occur at the cathode creating end products called faradic products. The production of hydroxyl ions (OH^-) locally reduces the oxygen concentration while increasing the pH. This environment favors osteoblastic activity (formation) and decreases osteoclastic activity (resorption) (Bodamyali et al., 1999).

On the other hand, the Inductive Coupling (IC) method exhibits its stimulating effect increasing and absorbing calcium from bone as an inactive signal to PTH (Luben et al., 1982; Spadaro & Bergstrom, 2002). Besides, the IC actively reserves intracellular calcium, increasing the levels of activated calmodulin, which improves the proliferation of osteoblasts. This activation differs from CC since intracellular calcium activation comes from the extracellular pathway (Brighton et al., 2001). Also, IC regulates the production of growth factors, including BMP -2,4,6,7, TGF-β1, and IGF-2 by osteoblasts (R K Aaron et al., 1999).

Therefore, DC, CC, and IC stimulate the osteological response by stimulating growth factors throughout the stimulation time (Gan et al., 2005).

Figure 22 shows the stimulation of growth factors by the different types of electrical stimulation methods.

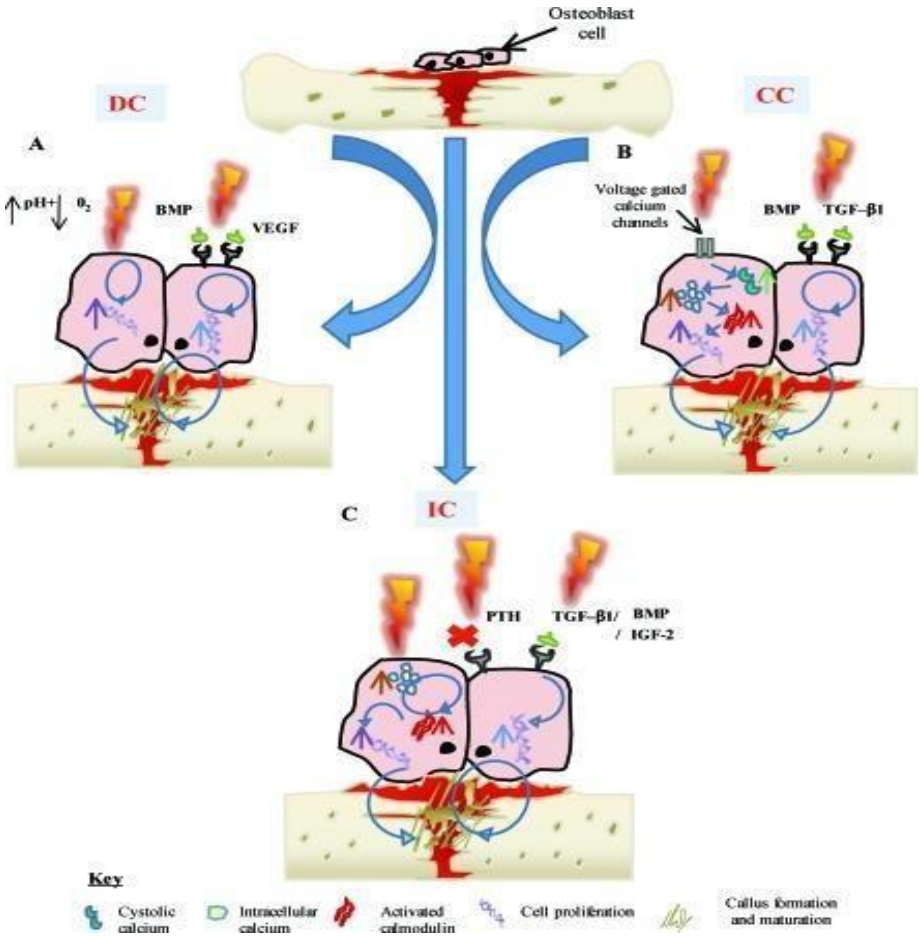

Figure 22. Proposed action mechanism of the different types of ES methods. Direct Current (DC) reduces the oxygen level and increases the pH by stimulating osteoblasts' proliferation that improves bone callus formation and maturation, leading to healing ***(b) Capacitive coupling (CC)*** *increases systolic calcium through voltage-gated calcium channels, which increases intracellular calcium, increasing activated calmodulin stores leading to cell proliferation and formation and maturation. of bone heat.* ***(c) Inductive coupling (IC)*** *causes an immediate increase in intracellular calcium that increases activated calmodulin stores, increasing cell proliferation and improving the formation and maturation of bone callus and, therefore, leads to healing.* By (M. Griffin & Bayat, 2011)

Direct current has been widely used to aid bone healing in spinal fusion, delayed unions, nonunion, and adjunct to bone healing in ankle surgery (Andersen et al., 2009; Steinberg et al., 1984).

Kane (1988) published the result of an *in vivo* clinical, randomized and controlled study on the use of ADC stimulation in high-risk patients undergoing posterior spinal fusions and risk factors such as obesity, smoking, and diabetes. The DC-treated group had an overall success rate of 81% compared to 54% for the control group. Similarly, Meril (1994) presented results of patients undergoing anterior and posterior lumbar interbody fusion with allografts and found that the overall success rate of the DC stimulated group was 95% compared to 75% in the control group, subsequently, in a group of patients who were smokers, the success rate was 92% versus 71% in the non- stimulated group.

Wu et al. (2015) experimented with accelerating a completely demineralized dentin collagen block's remineralization to regenerate the dentin microstructure of calcified collagen fibrils employing an electric field-assisted mineralization system in the absence of non-collagen proteins. The process accelerated by subjecting hydrogels current one constant power of 20 mA during electrophoresis, while in the control group was not applied any electric field. Subsequently, the collagen block was characterized, and it was visualized that the mineral particles created the structure of the original mineralized dentin, also that the intra and interfibrillar collagen fibrils were mineralized, concluding that the use of a direct current electric field was capable of promoting remineralization of the collagen matrix in dentin.

Capacitive coupling improves bone healing in spinal fusion, delated unions, and nonunion (Beck et al., 2008; Mäkelä, 1992). Londoño Abad et al. (1996) conducted a study in 12 patients with uncomplicated fractures of the lower jaw treated with

conventional intervention techniques and the help of electrical stimulation of 4-8.5 V and 20 mA with electrodes capable of adhering to the skin. After immobilization of the fracture, the patients received electrical stimulation for at least 8 hours per day for a term of 4 consecutive weeks. Radiographic and clinical controls were carried out during the study period and at the end of the treatment, concluding that all patients had clinical consolidation (absence of pain and movement at the fracture site) and a significantly shortened convalescence period.

Goodwin et al. (1999) conducted a prospective, randomized, double-blind clinical study on the use of CC stimulation as an adjunct to spinal fusions. Results showed that overall fusion success rates were 85% for the CC-stimulated group versus 65% for the control group. Scott & King (1994) showed that CC is effective as a treatment for pseudoarthrosis of long bones when treating ten patients, of which six healed at the end of treatment; these results differ from those of Beck et al. (2008) from a randomized controlled trial where the treatment group was assigned capacitively coupled electric field stimulation and the control group was assigned a placebo. The results showed no difference in healing time between the treatment and placebo groups; therefore, CC stimulation on the tibial stress fracture healing was not detected.

Sulca Buitrón (2019) proposed an experimental study to collagen's biomineralization with ES applied with CC in Simulated body fluid (SBF). Using demineralized rabbit bones, which were in their entirety to be submerged in EDTA; A dynamic system was implemented by placing electrodes on the outside surrounding the collagen that distributes the SBF liquid through the sample. Subsequently, the SBF was placed in a test tube, and copper electrodes around the tube connected to a dynamic CC signal as static, as seen in Figure 23. In the dynamic experiment, the electric field was directly applied to the collagen's surface, creating a strong electric field. In both dynamic experiments, the

electric field was applied on the glass tube, resulting in an indirect application and reducing the electric field's effectiveness, resulting in a very low polarization of the collagen. It was concluded that the dynamic experiment was more effective for mineralization than the static experiment.

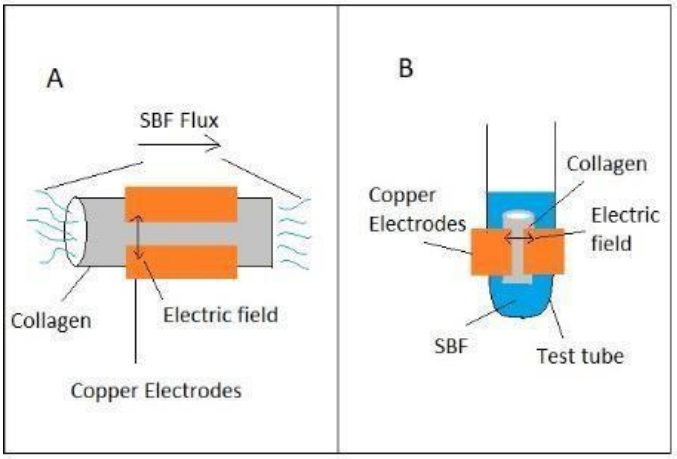

Figure 23. Experimental setup schematic (A) Dynamic experiment (B) Static experiment. Taken from (Sulca Buitrón, 2019)

Inductive coupling (IC), also known as a pulsed electromagnetic field, is used to treat bone healing with mixed results. Bassett et al. (1981) (1982) and Dunn & Rush (1984) demonstrated that IC is effective for the treatment of nonunion in long bones. The results of Borsalino et al. (1988), Mammi et al. (1993), and Traina et al. (1999) suggest that the use of IC improves the healing of tibial and femoral osteotomies. Betti et al. (1999) and Wahlström (1984) agree that recent fractures' healing can be improved through inductive coupling.

Contradictorily, the studies carried out by Eyres et al. (1996) showed that inductive coupling does not help in the treatment of osteoporosis and bone formation during limb lengthening, while for ankle arthrodesis, the results are incompatible and contradictory with those of (Saltzman et al., 2004; A Saxena et al., 2000; Amol Saxena et al., 2005).

Kim et al. (2019) suggest that in the absence of cells and applying a pulsed electrical stimulation, the transport of ionic fluids increases, and the diffusion of ionic precursors to collagen's internal surface producing bone mineralization. The study was conducted *in vitro*, and pulsed electrical stimulation was applied to tube-shaped collagen scaffolds extracted from chicken bones; the collagen was demineralized and subsequently mineralized in a modified SBF with 2.5 times higher concentrations of Ca^{2+} and P^{3-} than body plasma in a continuous flow. Pulsed electric stimulation was 40 mV and 1 Hz, similar to current bioelectrical generated by the walking human. During electrostimulation, the pulse generator supplied electrons to the cathode, negativelycharging adjacent areas of the cathode for a brief period attracting cationic precursors likeCa^{2+} and anionic precursors like HPO_4.

Pulsed stimulation mineralization was achieved only when collagen scaffolds were placed on the cathode since when placed on the anode, the mineralization levels were shallow. The study concluded that the local application of pulsed stimulation could improve mineralization by placing a cathode within the collagen scaffold, where the diffusion of precursor molecules is highly restricted Kim et al. (2019).

When no field is applied, the collagen molecules are arranged randomly; however, the collagen dipoles are oriented when an electric field is applied. The study carried out by Ficai et al. (2010) used a pulsed-field of 0.93 V/cm and a direct electric field to determine the degree of collagen orientation under a field's influence. The results showed that the electric field being similar to the pulsating electric field, the microstructure of collagen was oriented towards the charge.

In conclusion, the use of external electrical stimulation is a field widely studied due to its promising results; as has been explained, the technique's effectiveness is related to the stimulation of osteoblasts due to the increase in pH resulting from the application of current. Unlike mechanical fields, electric fields regulate the growth factor cascade and activate calcium and calmodulin channels that stimulate cellular functions.

Likewise, studies without cellular presence have determined that the electrical stimulus increases the transport of ionic fluids and the diffusion of ionic precursors to the collagen surface, producing its mineralization.

Since CC is an implantable technology, it sends electrical signals to the fractured area, so patient compliance is minimal, suitable for non-compliant patients; however, the technique is invasive, which carries the risk of infection and soft tissue reaction. In contrast, IC and CC coupling are beneficial options as they are non-invasive, require no surgery, and are painless, causing no problems for the patient to use the methods with ease.

Due to its characteristics, the application of external current for the remineralization of bone tissue is widely used to improve healing in various late and recent fractures, osteotomies, and bone grafts.

3.3 Magnetic fields: Magnitudes and method applications

In a body, the movement of electrons in its structure generates electric charges that generate a magnetic field. In the bone extracellular matrix, ionic transport through the sodium-potassium pump generates small endogenous currents; it is presumed to initiate the remodeling mechanism (Castro López, 2009).

When an external electrical current is applied, a magnetic field is generated that influences the human structure. Following this line, biomedical research in magnetic fields for bone remineralization has developed effective techniques a priori to maintain patient comfort.

The magnetic field B is measured in T and Gauss 10^4 G = 1T, and is generated by the current produced by electronic equipment (Guillen et al., 1985) consisting of a stretcher made mostly of stainless-steel, where the patient is laid down and the magnetic field passes through the tissues and can be up to four inches deep (Oliva Infante, 2018).

Magnetic fields can be generated by materials composed mainly of iron (Fe), cobalt (Co), or nickel (Ni) or by a variation of electric fields generated by a cylindrical conductive wire and is called an electromagnetic field (EMF) (Peng et al., 2019). EMFs used in cell regeneration include pulsed electromagnetic fields (PEMFs), alternating magnetic fields (AMFs), or rotating magnetic fields (RMFs) (Creecy et al., 2013).

The rotating magnetic fields (RMFs) increase the mineral density of the new tissue formed and enhance the integration of implants with host tissues. It has also been used for fracture-healing, spinal fusion, wound-healing, and osteoarthritis (Xia et al., 2018).

The application of static magnetic fields (SMFs) are indicated for long-term bone healing since it accelerates the proliferation, migration, orientation, and differentiation of osteoblast-like cells (Aydin & Bezer, 2011), and are divided according to the intensity

being ultra-weak (5 μT- 1 mT), weak (1 mT), moderate (1 mT to 1 T), strong (1-5 T) and ultra-strong (> 5 T) (Xia et al., 2018). It is presumed that low-frequency currents originate a more intense magnetic field than the electric field (Martín Cordero & García Delgado, 2009). So the accepted fields for osteogenesis are low frequency (up to 100 Hz), and intensity up to 100 gausses, the high frequency (up to 5000 Hz) are used in cases of pain, inflammatory disorders, and wound healing (Oliva Infante, 2018).

The magnetic fields are necessary to regulate biological systems since they are closely related to organisms' survival and evolution. The Earth's magnetic field is necessary to regulate biological systems since they result from ionic movements caused by electrical processes. It is hypothesized that decalcification is the product of the absence of the gravitational field inherent to the Earth; however (Guillen et al., 1985) stated that the absence of the Earth's magnetic field accelerates osteoclastic activity promoting tissue resorption.

(Boyan et al. (2002), Greenough (1992), Ito & Shirai (2001), Linovitz et al. (2002), Midura et al. (2005), and Shimizu et al. (1988) hypothesize that the mechanism by which magnetic fields improve bone healing increases blood circulation due to the dilation of blood vessels. Consequently, it creates a pool of oxygen and nutrients at the fracture site that promotes osteogenesis, helps the natural healers remove inflammation, allows calcium ions to adhere to the fracture site, and increases osteoblastic markers.

In vivo and *in vitro* studies suggest that SMFs and PEMFs can improve bone fracture healing and new tissue formation, below is a review of the research.

Yan et al. (1998) conducted studies implanting magnetized rods in the diaphysis of rat femurs. The surgical invasion produced a decrease in BMD; however, a local treatment with SMF by 12 weeks decrease the BMD reduction. The same procedure was used to

study the effect of SMF on bone formation in ischemic-induced rat femurs, and the results suggested that a 3-week SMF exposure can significantly decrease BMD reduction.

Kotani et al. (2002) carried out an *in vivo* and *in vitro* study to know the effect of strong order SMF (8 T) on the regulation of bone tissue shapes cell. *In vivo*, telopeptide-depleted bovine skin collagens pellets were implanted into male mice. Incisions were made in their backs, and the pellets were implanted subcutaneously; the mice were divided into two groups: the group exposed to SMF and the control group not exposed. The mice were exposed to a constant SMF stimulation maintained for 60 h, and the axis of the body of the mice was fixed in the direction of the magnetic field. Subsequently, the mice were euthanized. *In vitro*, they used mouse cultures of MC3T3-E1 osteoblast cells exposed to SMF stimulation for 60 h. After exposure, the cells were harvested, and cell counts were made per flask; also, the cells' orientation was determined.

The effects of exposure to strong SMF on the proliferation, orientation, and differentiation of MC3T3-E1 cells were not positive since there was no alteration. However, after the exposure, the cells of the cultures were oriented parallel to the direction of the magnetic field, unlike the control group, which was randomly oriented (see Figure 23), likewise, 14 days later, the exposed cells showed greater expression of ALP (alkaline phosphatase), an enzyme that plays an essential role in the mineralization of osteoid and whose level increases during bone formation. Also, the cells changed their rod-like shape and oriented in a direction parallel to the magnetic field.

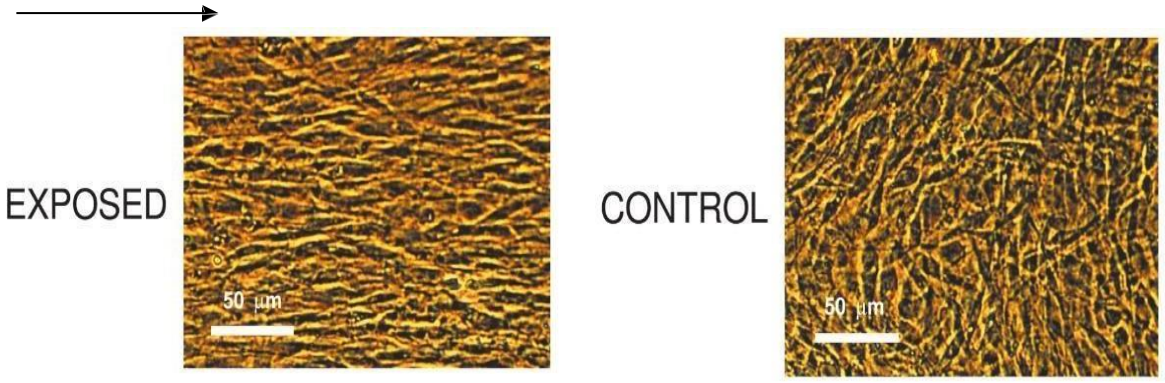

Figure 24. Effect of the SMF on the orientation of cultured MC3T3-E1 cells. The arrow indicates the direction of the magnetic fields. Taken from (Kotani et al., 2002)

To investigate the effect of SMF *in vivo*, 21 days after the exposure, BMP-2/collagen granules were collected, and radiological and histological analyzes were performed. X-rays revealed that SMF stimulated bone formation in and around BMP-2/collagen pellets. Furthermore, the bone mineral content (BMC) of the exposed group was approximately four times higher than that of the unexposed group. Histological examinations revealed that newly formed bone tissues replaced the granules. These results indicated that exposure to SMF could increase both bone mass and maturation *in vivo*.

Marcer et al. (1984) demonstrated the effectiveness of PEFMs in the treatment of non-union of the tibia and femur in dogs with stable fractures. The efficacy in the therapy for fractures was 75% -90%, depending on the fracture type. Likewise, Godley (1997) and Simmons (1985) ensure that electromagnetic pulses increase bone formation up to 85%. Ángel et al. (2002) conducted a clinical study in ten patients with diagnoses of stable, non-union, or delayed union fracture in long bones; the patients were divided into two groups (experimental and control), the experimental group was applied magnetotherapy at the fracture site at a dose of 20 Hz, 50% intensity for 20 minutes continuously for 3 months, meanwhile, the group of control, local heat was applied with a hot wet compress for 20 minutes and an isometric exercise program for 10 minutes. The results were favorable for magnetotherapy since patients with this treatment had a tendency to

consolidate up to 60% and an increase the metabolic activity in the muscle, improving recovery without any adverse effect. At the same time, in the control group, there was no complete union.

Xia et al. (2018) carried out studies of magnetic fields and magnetic nanoparticles (MNPs) on osteogenic enhancements, also Habib et al. (2020) developed magnetic nano-bone substitutes (MNBS) to study the combined effects with PEMF on human mesenchymal stem cell (hMSC) osteogenesis. The results concluded that PEMF combined with MNBS increases mineralization in a shorter time (see Figure 24).

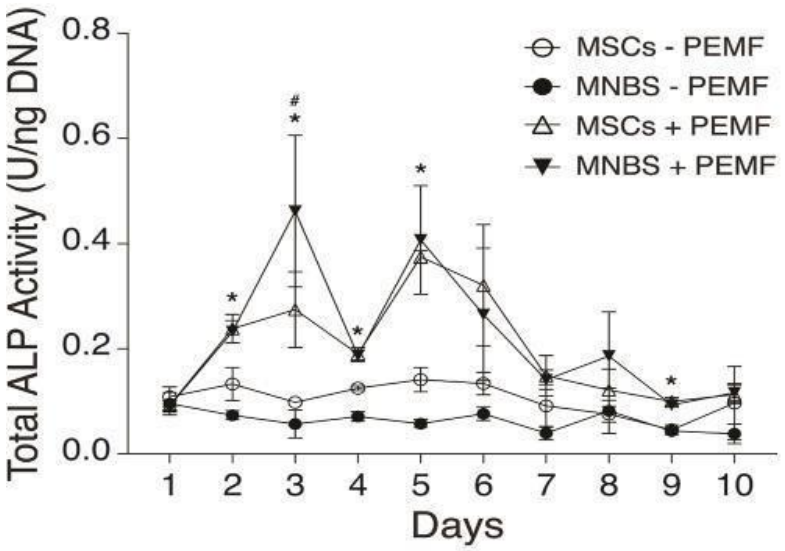

Figure 25. Effect of PEMF and MNBS treatment on total ALP activity. The samples were treated for up to 10 consecutive days. On days 2,3,4,5, and 9, total ALP activity was increased with PEMF treatment alone and was significantly greater than both groups without PEMF treatment. On day 3, the MNBS + PEMF treatment group's total ALP activity was significantly greater than all other groups.

Taken from (Habib et al., 2020)

The in vitro and in vivo results conclude that the use of the magnetic field promotes osteogenesis from the generation of nutritional substances that actively collaborate to return damaged tissues to normal. Also, it has a vasodilator effect acting on local

circulation with a powerful anti-inflammatory effect, so this technique is used mainly for the consolidation of fractures and degenerative diseases because it increases BMC.

Disadvantages of use include possible postoperative infection and rejection when implanting the magnetic material. Implanted magnetic materials can affect the results of medical examinations and treatments, and tissue fluids can corrode the material, requiring replacement in time (Peng et al., 2019). The use of magnetic fields to treat pathologies is contraindicated if the patient has a pacemaker, tumor pathologies, or is pregnant (Martín Cordero & García Delgado, 2009).

In conclusion, magnetotherapy is a non-aggressive or painful technique; it is performed without direct contact with the patient's skin (Guillen et al., 1985), widely used in physiotherapy. It constitutes a precise treatment that effectively helps inflammatory processes and, together with its high penetration rate, can generate electrical currents in bone tissue that incite osteoblasts to increase their bone production.

4. Discussion

The use of external fields in collagen remineralization promises significant advances in increasing bone density and decreasing mineralization time. The piezoelectric effect results from the mechanical stresses that occur naturally in the body, which allows the bone matrix to acquire an electrical polarization that serves as a signal for the osteoblasts that will consequently form new bone tissue.

Opinions are divided about the influence of HA on remineralization. (Hitmi et al., 1986) proposes that HA crystals present piezoelectricity due to the reorientation in the OH^- bonds, this influence being minimal compared to the organic component (Becker et al., 1977). The electrical currents generated during stress promote the bonds between collagen and hydroxyapatite (Pawlikowski, 2017) and induce hydroxyapatite to prevent excess water within the organic matrix (Ahn & Grodzinsky, 2009).

Studies by (Cowin et al., 1995; Rajabi et al., 2015) states that the mechanical field increases intracellular fluid present similar results (Turner, 2007) since the low-pressure fluid caused by a load causes a significant cellular response. Likewise, (Gan et al., 2005; Luben et al., 1982) concluded that by applying a CC, they increase transmembrane calcium transmission and increase calmodulin levels. Therefore, it is concluded that external fields' effectiveness lies in the increase in intercellular fluid, which activates different biochemical cascades.

The results suggested that dynamic loads promote bone formation in vitro (Hert et al., 1969) verified that dynamic mechanical loads allow extracellular fluid to increase, also (Sulca Buitrón, 2019) concluded that applying a dynamic electric current is more effective for mineralization than a static electric current.

(Ficai et al., 2010; Riggs et al., 1993) concluded that the alignment of the collagen matrix is oriented towards the applied external field, similarly; (Kotani et al., 2002) concluded that a strong SMF aligns the cultured cells of pre-osteoblasts to the field direction.

Moreover, both electric and magnetic fields create a natural pool of oxygen ions and increase the fracture site's pH at the fracture site (Bodamyali et al., 1999; Boyan et al., 2002; Ito & Shirai, 2001; Shimizu et al., 1988), which has not been proven in mechanical fields until now.

The mechanical fields help in the remineralization of the tissue, taking into account that the osteoblasts lose their mechanosensitivity after specific cycles, so the inclusion of rest periods between loads is necessary; the use of external mechanical fields is recommended for increase bone density, taking into account that when the bone is subjected to a greater load, it will fracture.

The electric field stimulates cascades of growth factors, especially bone morphogenic proteins (BMP). Due to its favorable results, the use of external electric fields is recommended in clinical pathologies such as pseudoarthrosis of long bones, for the healing of broken bones and to perform spinal fusions, taking into account that, dependingon the type of current, a placement surgery will be required of electrodes.

The use of magnetic fields has proven to be very useful in the recovery of fractures due to its vasodilator effect and its ability to stimulate the regeneration of bone tissue. By applying low-frequency magnetotherapy sessions, bone consolidation is stimulated, accelerating recovery. Its use resides in the palliative effect, so it is often considered a more practical alternative than the fields mentioned earlier because it is less invasive and with fewer contraindications.

5. Conclusion

The present work includes two essential parts.

The theoretical background exposes the components and processes to which the bone is subjected during its life. Bone formation begins at the embryonic period, where cartilage progressively hardens until it forms a complex tissue.

The hard tissue is formed from extracellular matrix and bone cells. The cells being responsible for synthesizing the collagen matrix and secreting hydroxyapatite precursor minerals. On the other hand, the extracellular matrix is the one that provides piezoelectricity to the bone since it is formed by both the organic matrix composed mainly of collagen and the inorganic matrix rich in minerals and mainly composed of hydroxyapatite.

The type V collagen, found chiefly in bone, is a quaternary protein made up of three polypeptide chains coiled in a left-handed way forming a right-handed helix; the basic unit is called tropocollagen, which forms organized fibers with spaces between them thatgive the bone flexibility. It is presumed that the piezoelectric effect of collagen is producedby the space between the fibers, which causes electrical polarization.

Hydroxyapatite crystals are deposited in the organic matrix during mineralization and providing the bone with hardness; the combination of hardness and flexibility gives the viscoelastic behavior necessary to resist excessive loads.

Although there are divided opinions, the present work maintains that endogenous currents generated during stress promote collagen-hydroxyapatite interaction bonds and influence piezoelectric behavior and maintain the necessary humidity within the extracellular matrix.

After its formation, every ten years, the skeleton is wholly renewed, so that bone remodeling is a continuous process where osteoblasts, osteoclasts, and a series of cascades of growth factors and proteins act, resulting in the replacement of old tissue by a new one. If enough energy is applied for the bone to exceed its yield point, a fracture will occur, and the bone will begin another process to replace the damaged tissue with new tissue with a similar shape and characteristics.

The second part, present the literary review about the effects of external fields on collagen piezoelectricity and collagen remineralization exposes the three external fields currently used in physiotherapy to treat bone fractures and pathologies.

Mechanical fields act on the bone, deforming it, which stimulates cellular communication and generates endogenous electrical charges that initiate the regeneration process. Because it is a non-invasive and easy-to-use technique, it is recommended in therapies to treat fractures and increase bone mass.

The electric fields used for therapeutic purposes are generated from cathodes connected to an energy machine whose objective is to simulate the endogen currents and stimulate the production of growth factors; The device can be surgically implanted, so it is recommended for patients who do not comply and in back pathologies and bone implants.

Magnetic fields improve blood circulation and promote the creation of a pool of growth factors at the site of the damage, making it a widely used technique in fractures, as it is easy to use and is not even necessary to touch the patient's skin. although it can also be an implanted device

In summary, this work presents a detailed and in-depth review of bone processes and how these can be influenced by applying external fields for the remineralization of bone

tissue, taking as a reference the piezoelectricity of collagen. This characteristic allows the bone to generate new tissue due to the application of external fields. With this information, external fields can be an excellent tool for therapeutic applications as they reduce recovery time by accelerating remodeling and mineralization processes, improving the patient's quality of life. Additionally, due to the versatility of the techniques, this study can be extended to the analysis of the dielectric properties of bone; these data would provide relevant information on the bone properties of most significant interest, which may contribute to improving and innovating in medical equipment rehabilitation and applications in other disciplines such as biomechanics.

Finally, an improvement to this work will consist of studies that combine two or more of the fields presented to know the mutual benefits of the techniques and an extensive review on the combination of magnetic fields with nano-bone substitutes (MNBS) to reduce the time of mineralization.

Abbreviations

AA Amino acids

$_t$RNA Transfer RNA

$_m$RNA MessengerRNA

RER Rough endoplasmic reticulum

ER Endoplasmic reticulum

HA Hydroxyapatite

BMP Bone morphogenetic protein

IGF Insulin-like growth factors

IL Interleukin

TNF Tumor necrosis factor

CSF Colony-stimulating factor

FGF Fibroblast growth factor

PDGF Platelet-derived growth factor

TGF- β Transforming growth factor- β

BMU Basic Mineral Unit

RANKL Receptor Activator for Nuclear Factor κ B Ligand

MMPs Metalloproteins

PKC Protein kinase C

PTH Parathyroid hormone

NPP1 Nucleotide pyrophosphatase/ phosphodiesterase

$CaPO_4$ Calcium phosphate

Pi Inorganic phosphorus

PPi Inorganic pyrophosphate

BMD Bone mineral density

SGP Streaming-Generated Potential

NO Nitric Acid

DC Direct Current

AC Alternating Current

ESElectrical Stimulation

ADCApplied DirectCurrent

CC Capacitive coupling

IC Inductive coupling

PEMF Pulsed Electromagnetic Field Therapy

ALK2 Activin receptor-like kinase-2

VEGF Vascular endothelial growth factor

SBF Simulated Body Fluid

EDTA Ethylenediaminetetraacetic acid

HPO_4 Phosphoric acid

PGE2 Prostaglandin

AMFs Alternating magnetic fields

RMFs Rotating magnetic fields

SMFs Static magnetic fields

ALP Alkaline phosphatase

BMC Bone mineral content

MNPs Magnetic Nanoparticles

MNBS Magnetic nano-bone substitutes

hMSC Human mesenchymal stem cell

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