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Therapeutic effectiveness of Hydroxyapatite Nanoparticles and Pulsed Electromagnetic Field in Osteoporosis and Cancer

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Abstract

The emergence of nanotechnology has had a profound effect on many areas of healthcare and scientific research. Several studies reported the importance Hydroxyapatite Nanoparticles in the biomedical field in general, and in emerging areas such as implants, drug delivery, cancer, composites, coatings, and ceramic materials in particular. On the other hand, low level Pulsed electromagnetic field (PEMF) therapy presents several potential advantages including non-invasiveness, safety, highly influential in the fracture repair process, lack of toxicity for non-cancerous cells, and the possibility of being combined with other available therapies. It has also been observed that the combined effect of these two can accelerate the osteogenic and anticancer activity in the osteoporotic and carcinoma cell lines respectively. The objective of this review is to provide a broad recount of the applications of PEMFs and Hydroxyapatite nanoparticles in osteoporosis and cancer and to then demonstrate what is further required for enhanced therapeutic outcomes.

Keywords: Osteoporosis, Cancer, Hydroxyapatite Nanoparticles, Pulsed Electromagnetic Field

Hydroxyapatite Nanoparticles (HApN):

In spite of the several developments in drug therapy and disease management, the research of pioneering methods to targets disease remains a very dynamic research field because of the array of unsettled therapeutic problems. Scientists are working now to create novel nanostructures that serve as new kinds of drugs for treating cancer, osteoporosis and Parkinson's. Presently [1-3], nanosized materials such as polymeric nanoparticles, gold nanoparticles, quantum dots, and liposomes are broadly used in the design of drugs, optical devices, imaging agent, gene-delivery systems, catalysts and biosensors. Over recent decade, because of biocompatibility, bioceramic based nanoparticles has enticed substantial attraction [4]. Out of several types of bioceramics, hydroxyapatite (HAp) is extremely important owing to its structural and chemical similarities to bone phosphates hydroxyapatite. Due to its bioactive and biocompatible properties, HAp has been found broad applications in the biomedical. HAp has been frequently used in bone tissue regeneration, enhancing osteogenesis and as an implanting material and coating material as well [5]. To enhance the bioactivity of implanting material, HAp is generally coated on the surface of the implanting materials. Furthermore, HAp is also a suitable material for carrying drugs to the targeted sites, or for a slow and unremitting release of drugs, which will eventually help in stimulating the growth of osteoblastic cells [5-7]. Moreover, HAp has shown to be used for numerous applications in the fields of protein chromatography, fertilizers, pharmaceuticals, water treatment, biomedicine owing to its stability with wide range of pH (4–14) [5, 8, 9].

Hydroxyapatite nanoparticles [HApN; $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] is a member of the apatite family that usually consists of Ca and phosphates. Due to its chemical composition and definite orientation [10, 11], HAp crystal comprised two binding sites, i.e. "Ca" sites (positive charged) and "PO₄" sites (negative charged) (figure.1). These two binding sites interact with their opposite charge protein/residues [12, 13].

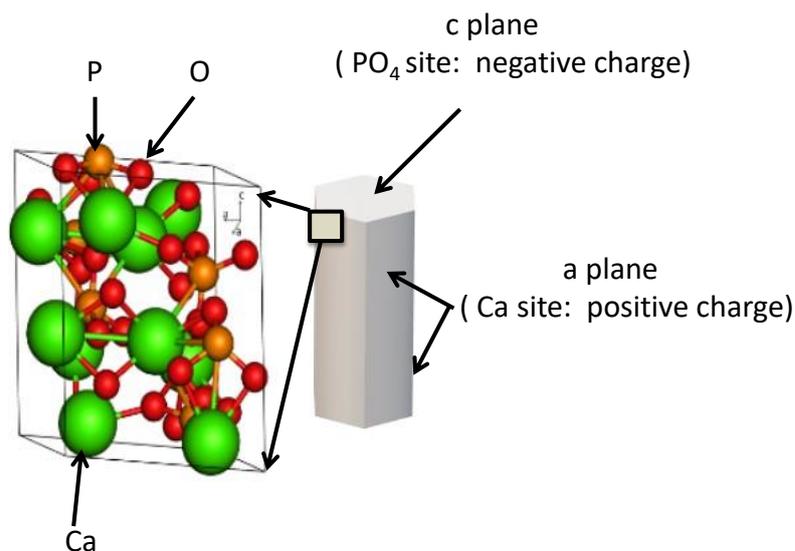


Figure 1: Hexagonal crystal structure of hydroxyapatite with “c” and “a” plane comprising Ca and PO₄ sites respectively. Molview 2.2 version software was used to draw the crystal structure of HAp.

Role of Hydroxyapatite Nanoparticles (HApN) in Osteoporosis:

Hydroxyapatite [HApN; Ca₁₀(PO₄)₆-(OH)₂] nanoparticles are chemically similar to the mineral component of bones and hard tissues in mammals. It is one of the few materials that are classified as bioactive, which means that it will support bone growth and osteointegration when used in orthopedics. Crystalline HAp particles are the typical mineral used in repair and may promote osteoblast cell adhesion [14, 15]. These particles are plate-shaped and hundreds of nanometers long [16, 17]. It is suggested that the basic inorganic building blocks of bone may be these nanosized apatite particles [18]. Tens to hundreds of these nanoblocks, in the collagen matrix, combine into self-assembled biomaterials that have remarkable physical and chemical features [19] such as unique mechanical strength, insensitivity to growth/dissolution, and flexible structure [20]. Thus, features of HAp nanoparticles may more closely approximate features of HAp during biomineralization. Therefore, HAp nanoparticles may promote the adhesion, proliferation and synthesis of alkaline phosphatase (ALP) in osteoblasts and lead to more rapid repair of hard tissue injury [21, 22].

It has been reported that HApN can be a better candidate in biomedical applications [23, 24]. However, the size dependent effects of these nanoparticles are not understood. Although conventional calcium phosphate materials have been well studied and shown to be biocompatible, the utility of smaller HAp particles as biomedical materials has received attention but the synthesis of HAp nanoparticles of well-defined size has been difficult to control [25, 26]. Therefore, new fabrication method should be used to engineer nanoparticles which can be useful for difference purposes [3]. Thus, questions concerning the mechanisms whereby cells detect and respond to HAp nanoparticles remain unresolved [27]. It is likely that HApN involve modulation of the interfacial forces that guide the organization of cytoskeletal elements and membrane receptor in cells. This, in turn, may modify intracellular signaling, binding sites of proteins and integrin signaling [28]. This hypothesis suggests that it may be possible to optimize the functionality of cells on the basis of size and other features of HAp nanoparticles. Information gained by investigations based on this hypothesis may also contribute to a more general understanding of biomineralization and biomaterials. The cell culture experiments on bone marrow mesenchymal stem cells (MSCs) showed improved cytophilicity of the nanophase mineral as compared with conventional HAp. Greater cell viability and proliferation of MSCs were measured on the nano HAp, remarkably for 20 nm sized particles [29].

In our earlier studies [30, 31], we have reported the effect of Silicon substituted hydroxyapatite nanoparticles (Si-HApN) on hind-limb suspended (HLS) wistar rat. A significant increase in BMD, Calcium (Ca), Phosphorous

(P), type I collagen, and ALP activity in femur and tibia in hind-limb bone of Si-HApN (20mg/250 g body weight) treated wistar rat (HLS+HAP) as compared to control (HLS). Our finding indicates that HApN has potential to encounter the bone loss.

An important but unsolved question is how the cells can recognize the particle size of HAp nanoparticles. It is well known that cells are inherently sensitive to their surroundings. It is suggested that the dissolution of HAp nanoparticles (small size and poor crystallinity) will increase concentrations of calcium and phosphate in the medium and thereby may alter the gene expression, alkali phosphate and proteins [32]. However, other findings contradict the dissolution of HAp in solution [29]. We observed significant increase in ALP activity, type I collagen and osteocalcin concentration in HLS + HAp group rats. It indicates that 20 mg/250 gm body weight of Si-HAp can induce and enhance the expression level of bone formation marker genes. Furthermore, the zeta potential measurements can provide the interfacial properties of the nanoparticles. HApN particles have a slightly negative zeta potential in water at pH 7.4 [33]. Since the electronic potential of cell membranes is known to be always negative; the positive or weakly negative particles are suitable for adsorption by cells. Another possible explanation is that the quantity of proteins adsorbed on particles positively correlates with their specific surface area [33, 34]. Nanosized particles adsorbed significantly greater amounts of proteins due to the presence of both positive ("Ca") and negative ("PO₄") sites and induce enhancement of subsequent cell adhesion and proliferation in hind-limb suspended wistar rat treated with HApN (HLS + HApN) and in hind-limb suspended wistar rat treated with both HApN and low level pulsed electromagnetic field (HLS + HApN + PEMF) group rats. It is suggested that PEMF helps to accelerate the process [30, 31].

Role of Hydroxyapatite Nanoparticles (HApN) in Cancer:

Exploring new materials for treatment and investigating the mechanism are important. With the development of nanometer technology, hydroxyapatite nanoparticle, a novel inorganic material, was found to be able to inhibit tumor cell proliferation. Hydroxyapatite [HAp; Ca₁₀(PO₄)₆(OH)₂] one of major inorganic components of mammalian bones, has been used extensively and successfully as bone defect filling material, as well as medicine bearer and coating material [35-37]. In recent years [2, 4, 5, 15, 38, 39], nanoscale hydroxyapatite (HApN), a novel biomedical material, which diameter less than 100 nm, has been reported that not only has better biocompatibility than HAP but also anti-cancer activity. It has been shown [40, 41] to inhibit the proliferation of various tumors, such as hepatoma, colon cancer, and osteosarcoma. Moreover, the antiproliferation effect of nano-HAp has been shown to be due to induction of apoptosis. However, the actual molecular mechanism of HApN -induced apoptosis remains unclear.

Several studies [42-45] on the effects of HApN on human gastric cancer SGC-7901 cells showed that HApN significantly reduced cell viability and induced apoptosis in SGC-7901 cells characterized by hypodiploid DNA contents, morphological changes and DNA fragmentation. The increase in apoptosis was accompanied with the increased expression of Bax, a pro-apoptotic protein and decreased expression of Bcl-2, an antiapoptotic protein, the decrease of mitochondrial membrane potential and the release of cytochrome c from mitochondria into cytosol. Furthermore, while HApN induced the activation of caspases-3 and 9, no activation of caspases-8 was observed.

Moreover, the exact internalization pathway into the cells represents the first necessary step towards the detailed investigation and optimization of the functional mechanism of HAp nanoparticles. In contrast to the organic and spheric latex particles, HAP nanoparticles are inorganic and needle shaped. Recent studies intensively documented the internalization of HAP nanoparticles into hepatoma cells via transmission electron microscopy and thus suggested phagocytosis as possible entrance pathway [46]. Since, the particle size can influence the pathway of internalization [47, 48], the increase of the particle size due to the nanoparticle agglomeration in the presence of organic medium has to be taken into account for the discussion of the internalization mechanism. The uptake of latex microspheres with a diameter smaller than 200 nm involved clathrin-coated pits while caveolae-mediated internalization became the predominant pathway of entry for particles of 500 nm in size [46].

Pulsed electromagnetic fields (PEMF):

In PEMF, a time-varying electrical current is passed through a conductor to produce a magnetic field based. Exposure to PEMFs in the 0–300 Hz range is a therapeutic tool widely used for the treatment of several diseases including osteoarthritis, postsurgical pain and edema, Parkinson’s disease and easing of vasodilatation and angiogenesis producing direct stimulation to excitable cells [49-51]. Initially it was it was thought that PEMFs induced forces through piezoelectricity [52-54]. Further, it has been reported by Funk *et.al* that electric fields exemplify forces at the surface of molecules, cell membranes and even the whole body, while magnetic fields penetrate deeper going inside the cell influencing chemical and biochemical reactions[55].

Role of low level Pulsed electromagnetic fields in Osteoporosis:

Pulsed electromagnetic fields (PEMF) have been successfully used for several decades to treat a wide range of bone disorders such as delayed and nonunion fractures, fresh fracture healing, prevention and reversal of osteoporosis[53]. The therapeutic effects of PEMF were first demonstrated in bone by Basset and colleagues[52], whose reports led to clinical trials and widespread commercial availability. Subsequently, PEMF has been demonstrated in blinded trials to be a safe and effective means of treating nonhealing bone fractures[56, 57].

Our device, known as an electrical bone stimulator, has been used to treat fractures and control osteoporosis. It emits a PEMF, which is applied to the chosen site of the long bone. The bone stimulator (Table 1, Figure 2) is connected to a pair of capacitor electrodes, which are applied over the skin.

Table 1 specification of Bone Stimulator:

Carrier Frequency:	14.0 MHz
Modulated Frequency:	16.0 Hz
Amplitude:	10 V (peak to peak)
Output Wave Shape:	Square
Electrode diameter:	1 cm
Average electric field between electrodes:	7.8 Volt / m



Figure 2: Pulsed electromagnetic field treatment on hind limb suspended wistar rat by Bone Stimulator.

Bone stimulator emits low level electromagnetic energy. In our earlier studies ,we have demonstrated that capacitive coupling of pulsed electromagnetic fields (CC-PEMF) exposure promote the restoration of the bone loss in tail-suspension induced [30], ovariectomy-induced [58, 59] and nurectomized osteoporosis [60] .Other studies also shown that PEMF is capable of preventing bone loss in animal models of disuse osteoporosis [52-54, 61, 62], tail-suspension-osteoporosis [63] and ovariectomy-induced osteoporosis [64-68]. Clinical investigations further confirmed that PEMF could help enhance bone mineral density and inhibit bone loss in human patients [69-71]. Despite this knowledge, we have to realize that the stimulus efficacy of PEMF was influenced by many factors, such as PEMF parameters [72], the duration of PEMF exposure [73] and the placement of PEMF generator [74] and others. However, due to the complexity of PEMF biological effects, there are still several other factors which have not yet been investigated.

Although, Pulsed electromagnetic fields (PEMF) are being used clinically to promote bone healing but relatively little is known about the mechanisms involved. EMF possibly directly alters ion binding and/or transport that leads to trigger the cascade of biological processes related to tissue growth and repair [75, 76]. In electromagnetic field, magnetic fields (MFs) penetrate deeper going inside the cell influencing chemical and biochemical reactions whereas electric fields (EFs) represent forces at the surface of molecules, cell membranes and even the whole body [75, 77, 78]. To date, despite having a somewhat concrete understanding, there is no conclusively defined mechanism of action. The main reason for such ambiguity as to how PEMFs act is the highly complex nature of the fracture healing process itself. Bone repair at molecular level comprised a number of signaling molecules like pro-inflammatory cytokines (e.g. interleukin-1 and interleukin-6), transforming growth factor-beta (TGF-b) super family and other growth factors (e.g., bone morphogenetic protein 2, insulin- like growth factor and growth differentiation factor) and angiogenic factors such as angiopoietin and vascular endothelial growth factors [79-81]. These factors work to induce differentiation and proliferation of mesenchymal stem cells (MSCs) into cartilage, fibrous tissue and bone.

It has shown that PEMF causes osteoblasts to produce paracrine factors, including transforming growth factor beta-1 (TGF-b1), prostaglandin E2 (PGE2) and bone morphogenetic protein-2 (BMP-2) [82-84]. The PEMF also induces human mesenchymal stem cells (MSCs) to produce TGF-b1 and PGE2, but only when they are cultured on calcium phosphate substrates in osteogenic medium and this is enhanced when the cells are treated with BMP-2 to induce osteoblastic differentiation [85]. These studies indicate that PEMF affects cells in the osteoblast

lineage to produce factors that stimulate osteoblast differentiation. The observation that TGF- β 1 also inhibits osteoclast activity suggests that PEMF may stimulate production of other factors that modulate bone resorption [86]. In addition to this, PEMF induces cells in the osteoblast lineage to produce osteoprotegerin (OPG).

The effect of PEMF was evident only when the cells were cultured on CaP disks, supporting the hypothesis that responses of osteoblastic cell to local and systemic factors, including mechanical and biophysical stimuli, are substrate dependent [86, 87]. Integrin expression is sensitive to surface properties, and there is a shift in integrin signaling when osteoblast-like cells are cultured on tissue culture polystyrene (TCPS) or calcium phosphate. The effects of PEMF on the OPG/RANKL (osteoprotegerin/receptor activator of nuclear factor kappa-B ligand) system were specific to OPG. No changes in RANKL mRNA or soluble RANKL protein were detected, regardless of the cell model examined. This suggests that PEMF acts via a signaling pathway that is distinct from pathways that mediate RANKL expression or secretion. Thus PEMF acts differently on cells in the osteoblast lineage depending on their state of differentiation [88]. As noted previously the stimulatory effects of PEMF on MSCs were greater in a culture environment that promoted osteoblast differentiation: growth on a CaP substrate in osteogenic media. In younger cultures, treatment of MSCs with BMP-2 to induce osteoblast differentiation also enhanced response to PEMF [86]. In more mature cultures, when more of the cells were expressing an osteoblast phenotype, no further enhancement of response was noted in BMP-2 treated cultures over that seen with PEMF alone [88]. However, BMP-2 stimulated cells were further stimulated by PEMF. BMP-2 has also been shown to increase OPG [89], suggesting that BMP and PEMF acted via different pathways.

In vitro PEMF studies focused on osteoblasts and its precursor, whereas the *in vivo* fracture environment is a complex interconnected system of different cell types. There exists a particularly intimate connection, both physical and biochemical, between blood vessels and bone cells in the multiple steps required for bone healing. Since all cells are exposed to PEMF during therapy, so, it is important to examine the effect of PEMF on both the paracrine and autocrine cell interaction between osteoblasts and endothelial cells *in vitro*. Osteoblasts and endothelial cells are in close approximation within the basic multicellular unit (BMU) of bone healing. In an attempt to understand the *in vivo* mechanism of effect of PEMF, it is therefore important to examine its effect on osteoblast–endothelial communication. Studies have demonstrated a reciprocal functional and regulatory relationship between osteoblasts and endothelial cells during osteogenesis that involves the pro-angiogenic vascular endothelial growth factor (VEGF) family, and in particular VEGF-A [90-92]. The clinical effect of PEMF on endothelial cells during bone healing may not be limited to a direct autocrine effect. Neighboring osteoblasts within the electrical field can affect endothelial cell proliferation in a paracrine manner through release of a soluble factor. It was found that PEMF acts by promoting angiogenesis through the coordinated release of FGF-2 and to a lesser extent several other vascular growth factors (Ang-2, TPO and EGF) [93]. This suggests that PEMF may facilitate healing by augmenting the interaction between osteogenesis and blood vessel growth.

The vast majority of bone marrow mesenchymal stem cells (BMMSCs) are present in the quiescent state (G0) while a small number of cells are actively engaged in proliferation (approximately 10% at S+G2+M). However, flow cytometric analysis revealed that PEMF treatment of chondrocytes led to significantly increased cell numbers in the S+G2+M cell cycle phase [94, 95]. The change of cell cycle progression of BMMSCs under PEMF treatment might be attributed to the alteration of cell membrane potential resulting from PEMF exposure. Previously [78], it is suggested that the possible mechanism of electromagnetic fields on cells is the forced-vibration of all the free ions on the surface of a cell's plasma membrane. Moreover, it was observed that voltage-gated delayed rectifier K⁺ current and Ca²⁺-activated K⁺ current channels were changed during progress from G1 to S phase, and functional expression of ion channels could regulate proliferation in undifferentiated rat mesenchymal stem cells [96]. These findings led us to infer that PEMF exposure changed the expression of ion channels and induced membrane hyperpolarization of BMMSCs and therefore resulted in the alteration of the cell cycle progression.

Role of low level Pulsed electromagnetic fields in Oncology:

The resistance of tumor cells to antineoplastic agents is a major obstacle during cancer chemotherapy. In fact, many patients do not respond to treatments and die due to metastasis, the main mechanism being drug resistance, the so-called multidrug resistance (MDR) phenomenon. The reason for this resistance is the expression of a membrane glycoprotein, named P-glycoprotein or P-170, that acts as a drug extracting pump, reducing the intracellular level of the antitumor agent [97, 98]. On exposure of PEF (typical parameters: 1kV/cm, 100µs), lipid bilayer in cell membrane could temporarily rearrange, leading to the formation of aqueous channels that are often called pores [99-101]. Such changes will make cell membrane more permeable to a variety of hydrophilic molecules, while there is no effect on the intramembranous organelles (e.g. nucleolus). After pulsing, in most cases, these pores will reseal without any damage to cell. This physical procedure of transient pores appearing at cell membrane is termed electroporation. The electroporation therapy, combining PEF with chemotherapy, has been applied for treating tumor [102-104], such as head-neck cancer, skin cancer, pancreatic cancer and liver cancer. In this way, exogenous molecules like HApN, cytostatics can penetrate more efficiently by exposure of the cells to a magnetic or electric field, which increases the cell membrane permeability. The higher uptake of drugs enhances cell killing [103]. On the other hand [105], it has been reported that pulsed magnetic fields (5.25 mT peak, 250 pulses/sec) enhance the potency of daunorubicin against KB-ChR-8-5-11 cells. It was suggested that the mechanism involved may be the inhibition of the efflux pump, P-glycoprotein. In addition, it was observed that 1.2 mT, 60 Hz magnetic fields partially block tamoxifen's inhibitory action on growth of human mammary tumor (MCF-7) cells *in vitro* [106]. Animal studies have shown that the use of magnetic fields can enhance drug delivery across biological barriers (rat abdominal skin), using benzoic acid as the drug candidate [107].

In vitro study of PEMF on various human cancer cell lines like breast cancer (e.g., MCF7, MDA-MB- 231 and T47D), pheochromocytoma-derived (PC12), and colon cancer (SW-480 and HCT-116) showed blockage of the development of neovascularization required for tumor supply, ant-proliferative and mitotic spindle disruption and induced genetic instability by reducing the stringency of the late-cycle (G2) checkpoint [108-111]. Moreover, study shows that PEMF treatment is target specific [112]. It has been reported that PEMF therapy (parameters: frequency of 20 Hz, intensity of 3 mT) with exposure time of 60 min/day for up to 3 days induce apoptosis in human breast adenocarcinoma cells (MCF7), but not to normal breast epithelial cells (MCF10). Though target specific behavior of PEMF is promising, but exposure was limited to 3 days only. Hence, long-term PEMF exposure needs to be evaluated in further studies considering the fact that effectiveness of PEMF is severely linked to the signal parameters, exposure magnitude, duration, signal shape, duration of treatment as well as the type of cells exposed to the magnetic field [113, 114].

In vivo study of PEMF on various mice or rat model like T-cell immunodeficient female nude mice, wistar rat and 23 SKH-1 immunocompetent albino mice showed tumor reduction, a significant decrease in serum AFP (a serum glycoprotein elevated in cancer patient and used as a carcinoma marker) level, significant pyknosis, shrinkage of tumor cell nuclei [115-117]. However, effectiveness of PEMF therapy reported to be proportional to the exposure period. In case of mouse models of breast cancer, mice exposed for 6 hrs for four weeks showed a significant reduction in tumor size, possibly due to the inhibition of angiogenesis that may suppress the formation of blood vessels in tumor tissues, reducing the tumor growth [118]. Moreover, to the certain extends PEMF treatment reported to be effective to treat patient suffering from advanced HCC, glioblastoma multiforme, colorectal, ovarian, pancreatic, prostate and thyroid cancer [119, 120].

Future trends in osteoporosis and cancer research with PEMF and HApN:

Combination of Nanoparticles and Pulsed Electromagnetic Field could be used as a positive countermeasure for osteoporosis and cancer disease. Earlier, we reported that a combination of low level PEMF and HAp nanoparticles has potential to control bone loss induced by simulated microgravity [30, 31].

It can be suggested that the future prospects to treat diseases lies somewhere in the combination therapy. It should be noted that combination may lead to development toxicities, which need to be evaluated along with its potential to control the disease. Research in next decade with PEMF and Nanoparticles will definitely add

information in area of medicine. Clinical studies have used PEMF therapy for both osteoporosis and cancer treatment. These studies show that PEMF therapy is safe and promising compared to other available cancer and osteoporosis therapies. On the other hand, due to its bioactive and biocompatible properties, HAp has been shown to be used as a countermeasure to prevent both osteoporosis and cancer. In the future, PEMFs with HApN could be used synergistically in the field of osteoporosis and oncology.

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