Effect of low level pulsed electromagnetic fields on induced osteoporosis in rat bone

Thesis Submitted to the Jawaharlal Nehru University
for the Award of the Degree of

Master of Philosophy

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July, 2000

Dedicated to my
Lovingly Mother.

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CERTIFICATE

The research work embodied in this thesis entitled, *Effect of low level pulsed electromagnetic fields on induced osteoporosis in rat bone,* has been carried out in the School of Environmental Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted so far, in part or full, for any other degree or diploma in any University.

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ACKNOWLEDGEMENTS

I wish to take this opportunity to express my deep gratitude and thanks to my supervisor, ***Prof. J. Behari*** *(School of Environmental Sciences, J. N. U., New Delhi.) for his continuous supervision, proper guidance and incessant encouragement during the course of my study. His valuable suggestions with critical approach have no doubt reformed my idea during the research work. I also wish to thank him for introducing me to this new emerging field of research.*

I am grateful to Dr. N. R. Jagannathan, (Head. of Department, Nuclear Magnetic Resonance, AIIMS, New Delhi.) for extending the necessary facilities that were required during my research work. I would like to thank especially Mr. Mahaveer, Mr. Mahesh, Mrs. Valsala and all members of NMR Dept. of AIIMS for extending their help and support.

I could not find enough words to express my deep sense of thanks to my labmates Paulraj, Uma, Sahu, Jayanand, Naveen and lab asst. Kamta for their untiring support, incessant encouragement and unconditional help. I think, words would cease to express thanks to my friends Preet, Kiran, Anju, Abhimanyu, Vijay and Amarnath for their encouragement

and help at crucial moments. Thanks are also due to my respected seniors Mr. Pramod, Mr. Sudesh, Mrs. Sawarna and Mr. Bala for their suggestions and help.

My sincere thanks are due to Mr. Rajesh Pathania and Mr. Girish Raj of Electron Microscopy Facility, AIIMS and Mr. Harsh and Mr. Nimayi of Kapil X-rays, Munirka, New Delhi for their timely help.

I am deeply indebted to my parents for their encouragement and blessings. I also thank all my family members - Brother, Sisters and in-laws.

My thanks are also due to:

- *Mr. Tyagi & Mr. Pandey - Animal House.*
- *Mr. Manish - Scientific Material supplier.*

Rajeev Lochan

Contents

Introduction:

Inside human body, bone has 3 main functions:

(i) It is a major organ for calcium homeostasis and a significant store of phosphate, magnesium, potassium and bicarbonate,

(ii) Bones provide mechanical support for the soft tissues and are levers for muscle action, and

(iii) Bone is the major site of hematopoiesis in the human adult.

To fulfill these functions, bone is continuously broken down and rebuilt and assumes the optimal structure for the minimum amount of material. When the break down and formation of bone are not in balance (break down is more than formation) there is bone loss. Most diseases of the skeleton are due to such an imbalance, resulting in systemic or local bone loss. viz.: osteoporosis, Hyper parathyroidism, periarticular bone loss in rheumatoid arthritis etc. Blocking the bone resorption can be helpful in all these conditions. The bone structure is maintained by a feedback system and increased mechanical strain stimulates bone growth and bone modeling and depresses bone remodeling. There exist minimum strain thresholds controlling modeling and remodeling, and these thresholds, or "set points," are generally referred to as the minimum effective

strain thresholds, which can be influenced by the hormones and nutrition (Frost, 1990; 1992).

Osteoporosis is a generalized or localized deficiency of bone matrix in which the mass of bone per unit volume is decreased in amount but normal in composition. It is characterized by the loss of mineralized bone, which results in structural failure. Bone is a living, constantly changing tissue, and normally there is a balance between the amount of old bone being removed and the amount of new bone replacing it. Osteoporosis is usually caused by accelerated resorption of bone, though decreased bone formation may lead to osteoporosis in such entities as Cushing's syndrome, prolonged steroid administration, and disuse or immobilization osteoporosis as in a casted extremity. Loss of mineral salts causes osteoporotic bone to become more lucent than normal. This may be difficult to detect, since about 30% of the bone density must be lost before it can be demonstrated as a lucent area on routine radiographs.

The major causes of generalized osteoporosis are aging and postmenopausal hormonal changes. As a person ages, bones lose density and become more brittle, fracturing more easily and healing more slowly. Many elderly persons also are less active and have poor diets that are deficient in protein. In postmenopausal osteoporosis there is a deficiency in the gonadal hormonal levels and decreased bone formation. Recent clinical surveys have

demonstrated that adult bone mass diminishes at a mean rate of 0.5% per year (Riggs et al., 1986), and it can reach a loss of 2% per year after menopause (Heaney, 1982). As the probability of fracture is closely related to a person's effective bone mass, a modality that could prevent or retard loss of bone might provide a substantial reduction in the incidence of skeletal morbidity.

Several prophylactic measures to prevent loss of bone are available; these include estrogen therapy, calcitonin, parathyroid hormone, bisphosphonates, vitamin D, prostaglandin E_2, supplemental dietary calcium and exercise. There also are treatment regimens that stimulate formation of bone, such as sodium fluoride and parathyroid hormone. Although these regimens have been shown to be effective in the treatment of osteoporosis, limitations, cautions, and dangers are inherent in their extended use. Even exercise, which provides the only endogenous stimulus to maintain normal bone mass, is potentially dangerous, as it may precipitate the fracture that it is supposed to prevent. The clinical potential for increasing bone mass or simply preventing bone loss, by alternative non-invasive means is therefore substantial.

Brighton et al. (1985a) concluded that a capacitatively coupled electrical signal, delivered through gel-coated electrodes, could largely reverse an established disuse osteoporosis due to neurectomy in the rat tibia. Histologic and mechanical data as well as biochemical evidences provided by Cruess et al.

(1983) reveal that electromagnetic fields can diminish abnormal rates of bone resorption and increase rates of bone formation. Bioelectric effects therefore appear to provide a link between mechanical stimuli and cellular behavior.

One non-invasive technique is the induction of low intensity electrical fields into the bone and surrounding tissues. Interest in the use of electricity to influence the regulation of bone mass was aroused, when Yasuda (1955) demonstrated that bone, under load, can generate an electrical potential. Relatively large potentials (1.0 to 1.5 millivolts), arising from the impact loading of wet bone, were subsequently measured by Bassett and Becker (1962), leading to the hypothesis that strain-generated electrical potentials, produced during functional activity, may in fact be the signal that is responsible for the regulation of specific cellular processes in bone (Bassett, 1968). More recently, the use of electricity as a treatment for delayed unions and non-unions of bone has received attention (Lavine and Grodzinsky, 1987). Behari et al (1991) showed that pulsed radio frequency electrical field can accelerate bone fracture healing in rats.

Clinton et al (1989) concluded that there is an effective window of pulsed electromagnetic fields in which bone mass can be controlled in the absence of function. This is achieved by comparatively short daily periods of exposure generated within a physiological intensity and frequency. Because of the

physiological characteristics of the signal, the electricity influences the behavior of cell populations that are responsible for bone-remodeling in the manner similar to what occurs in the normal milieu. These electrical fields can slow, inhibit or even reverse the osteoporotic processes that normally accompany disuse in animal model. But the results with only microradiograph images of turkey's ulna's shaft were not satisfactory as well as the method of giving stimulation was not totally non-invasive.

While these studies identified electricity or electromagnetic fields as a possible means of stimulating cellular activity in the skeleton, it remains unclear which aspects of the electrical field are responsible for regulating these cellular events (Brighton and McCluskey, 1988) and how the cells respond to these fields. Available research articles do not provide any information about the strength of bone after the treatment. Also there is only sparse evidence of a dose-response effect of electricity on inhibiting osteopenia (Brighton et al., 1985), none of which, to our knowledge, has focussed on the non-invasive use of electromagnetic fields.

Disuse Osteoporosis:

Disuse osteoporosis results from lack of stress and strain on the bone, as when parts of the skeleton are immobilized, removing normal stress and leading to osteoporosis. Ordinarily, disuse osteoporosis is relieved when the affected part is remobilized, but prolong immobilization can produce irreparable bone damage, especially in adults. Acute immobilization osteoporosis is frequently caused by paralysis or a body cast, especially in young patients. Without the stress on bone, osteoblasts are inactive and older bone is not replaced. Calcium withdrawal may be so rapid that hypercalcemia results. Excessive urinary excretion may lead to renal calculi. Administration of large doses of vitamin D and calcium to young patients in casts is harmful, inasmuch as it exaggerates the condition created by immobilization. The reduction of calcium intake and increase of fluid consumption relieve the hypercalcemia and hypercalciuria. With remobilization, the osteoblasts resume normal activity, and calcium again is laid down in newly formed matrix.

Disuse osteoporosis can be produced in laboratory animals by immobilization or denervation of an extremity (Martin and Gutman, 1978; Cruess et al., 1983). A number of immobilization methods have been used to

induce experimental disuse osteoporosis, each with its own procedural and/or histomorphometric advantages and disadvantages. Models of single leg immobilization include wrapping or casting the leg, tenotomy, neurotomy or neurectomy, and hemicordotomy. It has been shown that nonsurgical immobilization by wrapping or casting (primary disuse) produces rapid bone loss in cancellous and cortical bone (Chen et al., 1992; Li and Jee, 1991). Tenotomy, neurotomy or neurectomy, and hemicordotomy (disuse secondary to surgical intervention) also cause rapid decrease in bone mass in experimental animals (Turner and Bell, 1986; Weinreb et al., 1989; Yoshida et al., 1991). Unilateral sciatic neurotomy/neurectomy is a simple surgical procedure and has been used by many investigators with satisfactory results. In the neurectomized legs, some movements of the upper leg and knee joint persisted, but movements of the lower hind limb and ankle joint were completely abolished.

Zeng et al. (1996) found that;

(i) In cancellous bone, sciatic neurectomy inhibited age-related bone gain and lost bone mass which resulted in a reduction of trabecular bone volume (–46 %).

(ii) In cortical bone, the same operation only inhibited the age-related bone gain, but maintained the cortical bone area at the basal control level.

(iii) The cancellous bone loss after sciatic neurectomy was associated with the decrease in bone formation and the increase in accumulated eroded surface.

(iv) In both cancellous and cortical bones, sciatic neurectomy induced transient elevation in osteoblastic activity leading to an increase in bone formation rate during the first week after operation.

These end points declined below the control levels between 1 and 4 weeks, and then maintained at the control levels between 8 and 12 weeks postsurgery.

Aims and Objective:

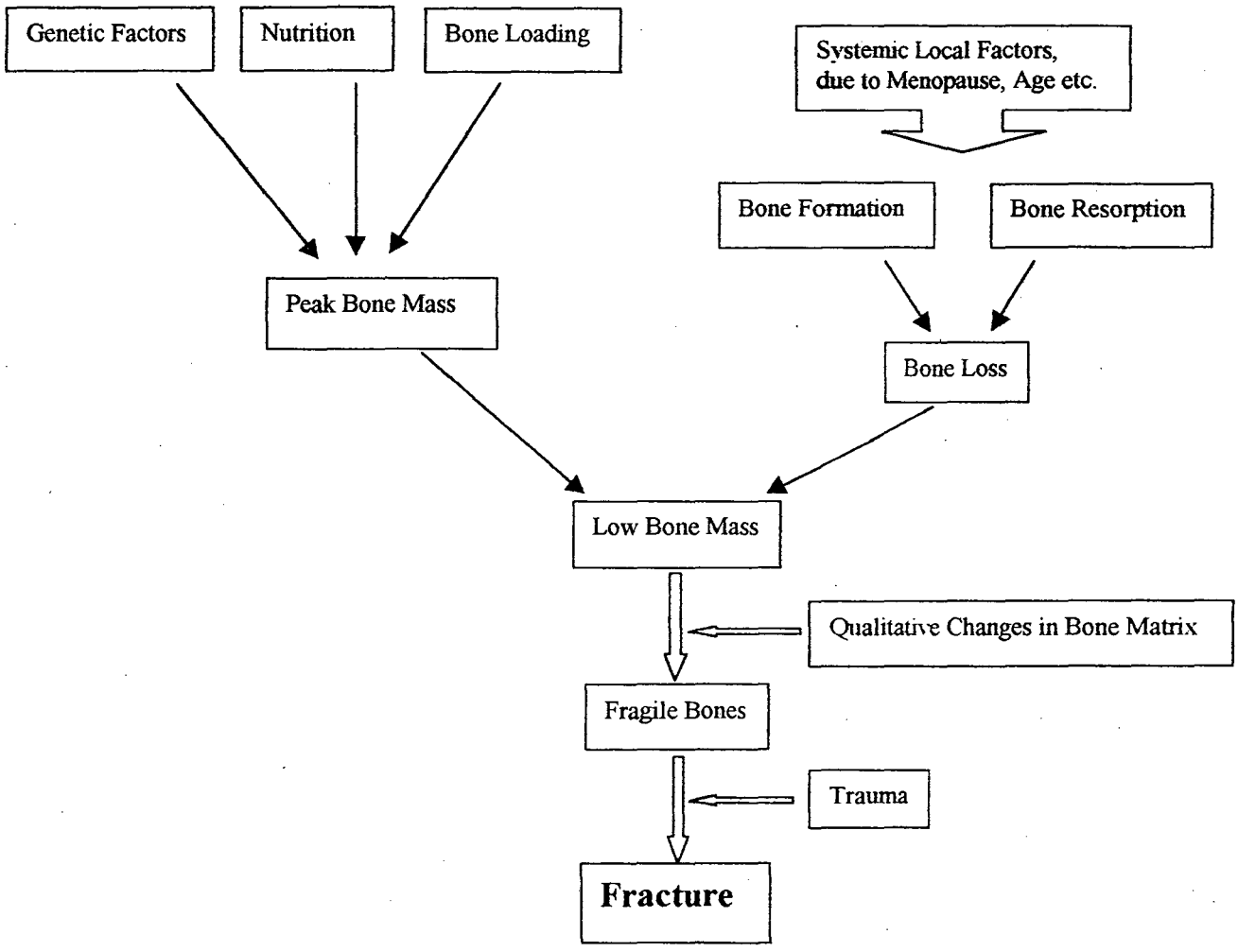
The objective of the present study is to specifically test and determine as to what extent a bone afflicted with osteoporosis responds to a non-invasive method of stimulation with amplitude modulated radio frequency waves. It is clinically relevant to investigate whether stimulation with modulated radio frequency waves can reverse aging related osteoporotic condition of human beings. The sciatic neurectomy technique by which osteoporosis is intended to be produced in an animal model results in the production of an osteoporotic bone essentially similar to chronically osteoporosis-afflicted bone seen in osteoporosis disease in human beings. Our experiment is not purely for academic reasons, but wholly for biomedical-cum-clinically relevant reasons. As there is no effective treatment of established osteoporosis, hence the efforts are on prevention of bone loss and fragility. The data derived from these experiments would provide significant information on as to what extent stimulation with amplitude modulated radio frequency waves could result in reversal (slowing down) of the process of osteoporosis and how the mechanism works. They will constitute a step in a right direction of translating the findings into trials to treat osteoporosis in the humans. Furthermore, details of the effectiveness of amplitude modulated radio frequency waves for treatment of osteoporosis will be revealed.

Related Works:

Osteoporosis is primarily a disease in bone density and predisposes to mechanical failure of the skeleton (Consensus Development Conference, 1987). Osteoporosis is probably the most common metabolic disorder of bone and may be drug induced, idiopathic, postmenopausal, or oophorectomy, but is mostly a result of aging. This is a clinical syndrome in which there is a decrease in cortical bone to levels below those required for mechanical support (Parfitt, 1983; Riggs and Melton, 1986; Escher et al., 1987; Cann et al., 1980). Osteoporosis results when the rate of bone resorption exceeds that of new bone formation (Meunier et al., 1980; Avioli, 1983) coupled with a defective osteoblast function (Marie et al., 1989). This causes an imbalance between bone resorbtion and bone formation (Meunier et al., 1980).

Riggs and melton (1983, 1986) suggested that involutional osteoporosis can be divided into two categories: postmenopausal (type-I) and age-related (type-II). This division is based on differences in patterns of fractures in different age group: type I osteoporosis (colles fracture) and type II osteoporosis (hip fracture). There are also differences in parathyroid function and mechanisms of fracture between these two groups (Riggs and Melton, 1990). In postmenopausal osteoporosis, increased bone resorption related to estrogen

Model for the pathogenesis of involutional osteoporosis.



deficiency is a major predictive factor. Age-related factors such as decreased calcium absorption, vitamin D deficiency, and overall inhibition of bone formation are important factors in type II osteoporosis. Nordin et al. (1990) estimate that more than half of the bone loss after menopause is age-related. At the tissue and cellular levels, menopause-related bone loss is characterized by increased bone resorption. Decreased bone formation is probably the main cause of age-related bone loss.

Genetic factors play an important role in the pathogenesis of osteoporosis. Studies in twins and in normal families indicate that between 75% and 85% of the variance in bone mineral density (BMD) is under genetic control (Soroko et al., 1994). Environmental factors such as calcium and vitamin D intake can modify the effects of vitamin D receptor gene (VDR) alleles on calcium metabolism and it has been suggested that the relationship between VDR genotype and bone mass may be masked in populations with a high calcium intake (Ralston, 1997). Genetic factors in bone cells mainly determine bone's material properties (except in osteomalacia), whereas modeling and remodeling mainly determine the "mass" and architectural factors in a bone's strength (Martin and Burr, 1989). As loads on bone increase during growth, modeling tries to increase bone strength to keep strains from exceeding the modeling threshold. That would keep them well below the microdamage and its effect on

bone fragility (Kimmel, 1993). Most aging humans lose bone because of their gradually decreasing muscle strength and women may have less bone strength and mass than men because they have weaker muscles (Frost, 1998). Studies on genetic basis of osteoporosis are in their infancy but have clinical relevance, not only in identifying informative polymorphisms, which could be of value in the assessment of osteoporotic fracture risk, and predicting response to treatment, but also in uncovering new molecular targets for therapeutic intervention in the prevention and treatment of osteoporosis (Ralston, 1999).

The final clinical outcome of the osteoporotic process is a fracture, which can occur as a result of a minimal trauma or even spontaneously. The majority of painful episodes of osteoporosis are not due to the osteoporotic process itself but are associated with fractures, particularly vertebral fractures. Osteoporosis is a serious medical problem and a major cause of morbidity in the elderly. There is no effective treatment of established osteoporosis, hence the efforts are on for prevention of bone loss and fragility (Kleerekoper and Krane, 1989). Increased bone mineral density has a protective effect against the effects of osteoporosis.

Bone marrow cells include hematopoietic cells and a heterogeneous nonhematopoietic component including fibroblasts, endothelial cells, reticular cells, adipocytes, and macrophages, which together comprise the marrow stroma (Bresford, 1989). The rodent bone marrow culture system consists of mostly

nonadherent cells of hematopoietic origin and a smaller adherent cell population, which includes stromal fibroblastic cells forming colonies with high alkaline phosphatase (ALP) activity, subsequently leading to mineralized nodule formation (Owen et al., 1987). These adherent cells therefore, are thought to include the ultimate precursors of osteoblasts (Richard et al., 1994). On the other hand, osteoclasts arise from hematopoietic mononuclear cells in the bone marrow (Roodman et al., 1985), while the cell of origin for the osteoclast in bone marrow is still a matter of controversy, the most likely stem cell is contained in a colony forming unit for granulocytes and macrophages (CFU-GM) (Mundy, 1993). It is also clear that rodent bone marrow cells develop into osteoclast-like multinucleated cells under optimum stimuli, such as the presence of $1,25(OH)_2D_3$, parathyroid hormone (PTH), and prostaglandin E_2 (PGE_2) (Suda et al., 1992).

The cells that carry out bone resorption, as mentioned above, are the osteoclasts. Bone resorption is a necessary process for the normal development of the skeleton, for its adaptability as well as its maintenance. This cellular process is essential in the growth, remodeling and repair of bone and in normal conditions it is tightly coupled with the process of bone formation by osteoblasts (bone formation cells). The osteoclast is a highly motile cell that attaches to and migrates along the surface of bone, mostly composed of the interface between

bone and bone marrow. The osteoclast is a multinucleated cell (mononuclear also) that is formed by the synchronous fusion of mononuclear precursors derived from the bone marrow. The osteoclast attaches to mineralized bone matrix that it is going to resorb by forming a tight ring-like zone of adhesion - the sealing zone. This is done by the bundles of actin filaments (Podosomes) with fimbrin, α-actinin, gelsolin and integral membrane protein with RGD sequences. The space contained inside this ring of attachment and between the osteoclast and the bone matrix constitutes the bone-resorbing compartment. The osteoclast synthesizes several proteolytic enzymes, which are then vectorially transported and secreted to this extracellular bone-resorbing compartment. Simultaneously, the osteoclast lowers the pH of this compartment by extruding proton across its apical membrane (facing the bone matrix). The concerted action of enzymes including cystein-proteinases, cathepsins B, L (Delaisse et al, 1991) and C, and also several classes of enzymes such as phosphates, and matrix metalloproteinases and the low pH in the bone resorbing compartment leads to the extracellular digestion of the material and organic phases of the bone matrix. After resorbing to a certain depth, determined by the mechanism, (that remain to be elucidated) the osteoclast detaches and moves along the bone surface before reattaching and forming another resorption lacuna, usually in close proximity to the first one. In the process, a certain volume of bone matrix is removed (only

replaced) under normal circumstances, by newly formed matrix a few days later. Calcium, phosphate and other components of the matrix (most of which have been completely digested, but some of which have been only mobilized during this process), are either be eliminated and serve locally as messengers, or be utilized at sites where bone formation and mineralization occur, or be used to maintain the proper ionic concentrations in the extracellular fluids.

One of the main cytochemical characteristics of the osteoclast is its enrichment in lysosomal enzymes. The high concentration of lysosomal enzymes in the osteoclast is not due to the presence of phagocytic structures such as secondary lysosomes. Instead, these enzymes are found, for the most part, in elements of exocytic pathway (Baron et al.1988). Their presence is demonstrated in the endoplasmic reticulum, golgi complexes and in numerous transport vesicles of the osteoclasts. Thus, the enrichment in lysosomal enzymes does not reflect a high phagocytic activity, rather a high biosynthetic activity.

During the active bone resorption, the buffering capacity exerted by solubilized bone salts, is likely to cause a gradient of pH extending from the most acidic zone, in the immediate vicinity of the ruffled border and its proton pumps, to a more neutral zone, deeper in the resorbing lacuna and towards the interface between mineralized and demineralized matrix. The higher pH in these regions would both favor the activation of procollagenase by the lysosomal

cystein-proteinases, because this process is more efficient around pH 6 than at lower pH and render the collagenolytic action of collagenase predominant, because cystein-proteinases are quite inefficient near neutral pH (Baron et al., 1993). Thus it would then be cooperative action of a set of acidic and neutral pH classes of the enzymes that leads to a complete degradation of extracellular matrix at the resorbing site.

Estrogen deprivation occurring after menopause or after oophorectomy causes increased bone turnover and accelerates bone loss (Gotfredsen et al., 1987; Wronski et al., 1988a; Longcope et al., 1989; Nordin et al., 1990). Studies of young women with severe estrogen deficiency resulting illness (Rigotti et al., 1984) or excess physical activity (Drinkwater et al., 1984) have documented a marked reduction in axial and appendicular bone density. Estrogen replacement therapy following oophorectomy is found to preserve bone mass (Lindsay et al., 1980; Genant et al., 1982). A decline in plasma estrogen levels causes an increase in bone sensitivity to the resorptive action of parathyroid hormone (Orimo et al., 1972; Gallagher et al., 1980). The estrogen effect may be mediated by calcitonin, a hormone secreted by C cells of thyroid, which lowers plasma calcium and phosphate and inhibits bone resorption (Greenberg et al., 1986). It has been demonstrated that estrogen therapy increases 1-25$(OH)_2$ vitamin D_3 serum levels (Stock et al., 1985)), and calcium absorption across the

intestine (Heaney et al., 1978). Goulding and Gold (1990) have shown that estrogen deprivation associated with administration of luteinizing hormone releasing hormone antagonist caused a significant decrease in total body calcium in rats. Estrogen therapy reduces bone remodeling to premenopausal levels, reduces the rate of skeletal tissue loss, and reduces the risk of fractures (Lindsay 1988). Long-term estrogen therapy must be administered cautiously, because it has been associated with increased risk of endometrial carcinoma (Jick et al., 1979; Hulka et al., 1980). Combined estrogen and progesterone therapy reduces that risk and is therefore recommended as the most suitable long-term therapy for postmenopausal bone loss prevention (Christiansen et al., 1981; Riis et al., 1987a). Progestogens, administered to postmenopausal women, decrease urinary calcium and hydroxyproline excretion (Selby et al., 1985). Barbagallo et al. (1989) have concluded that the long-term consequence of oophorectomy in rats causes a significant decrease in bone mass and the administration of progesterone is very effective in preventing the subsequent development of osteoporosis. However, the effects of hormone replacement therapy persist only as long as the therapy, and are lost when the steroids are discontinued. In addition, side effects may be of concern.

The incubation of the cells in the presence of calcitonin or in the presence of high extracellular calcium (2-4mM) lead to an increase in intracellular calcium concentration and an inhibition of bone resorption.

Teti et al. (1991) and Zaidi (1990) have suggested a model by which -

(i) Elevation of Ca_i leads to inactivation of the osteoclasts and conversely activation of the cell may, in some circumstances be associated with a decrease in Ca_i,

(ii) Elevation of Ca_i may be achieved by legend-induced (calcitonin, platelet activating factor, RGD proteins) or ion induced mechanisms, and

(iii) Elevation of Ca concentration in the sealed off extracellular bone resorbing compartment would lead to opening of the Ca sensor, elevation of Ca_i, inactivation and attachment of the osteoclast and thereby diffusion of the mobilized extracellular calcium into the extracellular fluids.

Calcitonin acts primarily to produce anabolic effects in bone by suppressing osteoclastic activity (Chambers, 1985). The mature osteoclast is directly and negatively regulated by calcitonin, for which the cell expresses a high number of receptors in most species (Nicholson et al. 1986, 1988). Calcitonin affects the cytoskeleton and attachment structures (Dempster et al. 1987; Lakka Korpi and Vaanan 1990), the secretion of enzymes (Baron et al. 1990b), the motility and the volume of the osteoclast (Chamber et al. 1984) and

some ion transport systems (Chakraborty et al. 1991, Malgaroli et al. 1989). Calcitonin appears to stimulate osteoblasts (Azria, 1989) and to promote internal calcium absorption (Chesnut et al., 1984). The use of Calcitonin in combination with glucocorticoids produces a more rapid, profound and persistent hypercalcemia activity (Mundy and Martin, 1982). It is suggestive in patients with myelome and other hematologic neoplasma, where glucocorticoids have a direct antimurol activity; however, it is of little help in hypercalcemia associated with solid tumor (Behari, 1994). Moreover, there is some confusion over the optimal dose of Calcitonin for the prevention of osteoporosis, and it has not yet been established whether Calcitonin prevents the loss of trabecular and cortical bone to the same content (Reginster, 1993).

Bisphosphonates is capable of increasing spinal bone mass 2 to 5% in postmenopausal women or elderly individuals (Gallagher, 1988; Storm et al., 1990; Harris et al., 1991; Reginster, 1992). They prevent fractures despite the limited increase in spinal bone density indicating that higher bone quality might have been achieved in therapy (Overgard et al., 1989; Watts et al., 1990). The effects of bisphosphonates compounds in preventing bone loss has been reported by a number of authors using various immobilization models (Michael et al., 1971; Schoutens et al., 1988; Thompson et al., 1990), but none of these experiments included testing of the mechanical properties of the bone.

A systematic infusion of Prostaglandin E_2 (PGE_2) *in vivo* increased the calcified area of rat bone (Jee et al., 1985). PGE_2 is produced in large amounts by solid tumors (Seyberth et al., 1975) and has been reported to enhance bone formation (Chyun and Raisz, 1984). It has been reported that the initial rapid bone resorption occurring after oophorectomy and disuse can be retarded by cyclooxygenase inhibitors (inhibitors of prostaglandin synthesis) (Lane et al., 1989; Thompson and Rodan, 1988). PGE_2 administered in rats has been shown to:

(i) Increase in trabecular bone mass by forming new woven trabeculae (Mori et al., 1990);

(ii) Increase cortical bone mass (Jee et al., 1990); and

(iii) Increase Haversian remodeling, presumably by increasing activation (Jee et al., 1990).

PGE_2 acts as a first messenger in the transduction of mechanical usage to bone cells. Increased mechanical strain causes PGE_2 production, which results in osteoblastic proliferation (Somjen et al., 1980; Binderman et al., 1984). Lin et al. (1994) have concluded that combination of PGE_2 and Risedronate (Ris) is more anabolic than PGE_2 or Ris alone when endochondral ossification is active, but PGE_2 with Ris is no more anabolic than PGE_2 alone in old bone without endochrondal ossification.

Growth hormone has similar effects on bone, as does PGE_2. It increases Haversian remodeling, presumably by increasing activation and increases bone formation (Harris et al., 1972; Aloia et al., 1976). Growth hormone also increases the efficiency of calcium absorption and decreases calcium excretion. The anabolic effects of growth hormone on the skeleton are thought to be mediated by insulin-like growth factor I (IGF-I) (Isgaard et al., 1988). The action of PGE_2, growth hormone, and PTH are different from the action of estrogen in one important aspect: estrogen tends to slow down remodeling, while PGE_2, growth hormone, and PTH tend to activate and accelerate remodeling. This accelerated remodeling is commonly associated with disuse. Yet, each of these agents has the ability to cause anabolic effects on bone.

Calcitriol or 1-25 Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which has a profound influence on the differentiation of bone marrow precursors into osteoclasts (Billecocq et al., 1990b; Takahashi et al., 1989), induces the expression of several phenotypic and functional markers of the osteoclast: Multinucleation and TRAP (Roodman et al., 1985), Calcitonin receptors and VNRs (Bellicocq et al., 1990a), Sodium pump (Billecocq et al., 1990a) and proton pumps (Kurihara et al., 1990). The overall effects of $1,25(OH)_2D_3$ on mineral metabolism (Reichel et al., 1989; Holick, 1995; Haussler et al., 1998; Norman, 1998) may be summarized as follows:

(i) Increased intestinal calcium absorption, leading to a rise in serum;

(ii) Decrease of serum parathyroid hormone (PTH) level (both through direct inhibition of PTH secretion from the parathyroid gland and through indirect inhibition of PTH secretion by the raised serum calcium level);

(iii) Decreased bone resorption due to a reduction in the PTH-mediated bone resorption; and

(iv) Under certain conditions, increased bone formation.

Calcitriol has been shown to increase total body and spine bone mineral density, and to reduce the incidence of new vertebral fractures (Gallagher et al., 1989), But the window of efficacy is quite narrow. The physiologic response to inadequate calcium is presumably an increase in PTH secretion, which subsequently restores and maintains blood calcium levels at the expense of the skeleton. Circulating levels of PTH rise during normal human aging, particularly in women (Marcus et al., 1984). Moreover, a normally observed nocturnal rise in PTH secretion is temporally associated with increased biochemical evidence for bone resorption. When administered in an intermittent fashion, PTH can be shown to exert impressive anabolic effects on trabecular bone (Reeve et al., 1980). PTH prevents bone loss in ovariectomized rats (Hock et al., 1988; Liu and Kalu, 1990; Takahashi et al., 1991) and restores lost bone in osteopenic ovariectomized rats (Liu et al., 1991; Shen et al., 1992; Kimmel et al., 1993;

wronski et al., 1994). Wronski and Yen (1994) concluded that in contrast to estrogen and bisphosphonate risedronate, PTH stimulates cortical bone formation and augments cortical bone mass in the tibial diaphysis of overiectomized rats. Mosekilde et al. (1994) examined the effects of PTH, estrogen and bisphosphonate (Risedronate) and the combination of PTH with antiresorptive drugs on vertebral bone mass and biochemical competence in a rat model and suggested PTH alone or in combination with antiresorptive agents as a promising therapeutic regimen for postmenopausal osteoporosis.

Bone is the major reservoir for calcium, accounting for 99% of total body calcium. The role of calcium in the treatment of osteoporosis appears to be due to its ability to decrease bone turnover (Elders et al., 1994; Elffors et al., 1994; Riggs et al., 1996) and thereby decrease skeletal losses. It seems likely that this is related to the small increments in serum calcium (Dawson-Hughe, 1996) and the resulting decrease in the activation of bone turnover. Markers of resorption and formation decrease by about 10%-20% (Elders et al., 1994) and remain suppressed during treatment. In postmenopausal women, urine calcium excretion increases markedly after starting treatment with additional calcium (Nilas et al., 1984). The effect is, however, attenuated with time and after 2 years, reaches pretreatment values. The difference in adaptation between pre- and postmenopausal women is that, in the latter, bone loss persists, albeit at a

slower rate, because the balance of resorption and formation at remodeling sites remains unchanged. Various considerations suggest that there are considerable uncertainties concerning the relationship of lifetime calcium intakes and bone mass or fracture risk. The effects of calcium in older women appear to be greater than women at or near the menopause in terms of the response in bone mineral density (Ettinger et al., 1987). A controlled, perspective study in the elderly in sheltered accommodation in France reported that calcium (1.2 g per day) and vitamin D (800 IU) significantly decreased the frequency of hip fracture in women >75 years (Chapuy et al., 1994). O'Brien et al. (1998) concluded that calcium intake alone is not capable of maintaining calcium balance in adult women from osteoporotic families, and optimum calcium intake should be provided to those young girls, having a family history of low bone mass to attainment of peak bone mass and the prevention of future osteoporosis. But there is still some uncertainty as to the long-term effects on bone mineral density and therefore on fracture frequency. A major advantage of calcium is that it is free from significant side effects and easy to take and can be given over a lifetime. Its major disadvantage is that it appears to be less effective than many other available bone-active agents (Kanis, 1999).

The beneficial effect of exercise on bone mass is brought about when dietary calcium was supplied adequately. In young bones, both bone modeling

and bone remodeling are active, whereas in adult bones, bone modeling is greatly slowed and bone remodeling is predominant (Baron et al., 1984). Barengolts et al. (1993) reported that exercise had beneficial effects on the bone mineral of the tibia and the femur during the early stage after ovariectomy in adult rats and improved the bending strength of intact femur without any effect on bone mineral of the tibia and the femur in sham-operated rats. Iwamoto et al. (1998) concluded that the exercise therapy under controlled calcium intake cannot only stop decreasing bone mass and mechanical strength but also increase them in patients with bone loss resulting from menopause and inadequate calcium intake. However, even if exercise results in positive effects on the skeleton, the optimal exercise detail, i.e., intensity, duration, frequency, and the period of the exercise which effectively increase bone mass or decrease bone loss in postmenopausal or elderly women, has not been determined yet. The inconsistent results of the effects of exercise on bone are probably due to the modifying effects of calcium intake on bone mass (Specker, 1996).

Fluoride is known to stimulate osteoblast proliferation (Farely et al., 1983). Initially, primitive woven-like bone is formed but later the bone is remodeled again in a normal way, creating lamellar bone organized in so-called packets (Eriksen et al., 1985). NaF (Sodium Flupride) has been identified as a useful therapeutic agent (Heaney et al., 1989). NaF has an anabolic effect on

trabecular bone mass, particularly in the spine (Aaron et al., 1991). Turner et al. (1992) showed that incorporation of fluoride into bone mineral has a dose-specific effect on bone strength, so that bone strength followed a biphasic relationship with bone fluorides. Some investigators have found that NaF increases the appendicular trabecular bone mass (Dambacher et al., 1986; Resch et al., 1993), whereas others have found no effect or even a decrease in predominantly cortical bone mass (Hodsman and Drost, 1989; Pouilles et al., 1991; Kleerekoper and Mendlovic, 1993) suggesting that an improvement in axial bone density may be at the expense of cortical bone (Hodsman and Drost, 1989; Riggs et al., 1990). Furthermore, several studies have shown structural abnormalities or mineralization defects in the bone formed during fluoride administration (Kragstrup et al., 1989; Lundy et al., 1989; Boivin et al., 1993), thus suggesting that the increase in bone mass is not necessarily followed by an improvement in bone quality. Sowers et al. (1991) reported a significantly higher risk of wrist, spine, or hip fractures in a community with high fluoride water content compared with the control community. These findings, along with those of Turner et al. (1992), suggest that there is a concentration threshold above which fluoride may become detrimental to bone strength and debate has been arisen as to determining the level of toxic dose. There have also been reports of an increased incidence of nonvertebral fractures during fluoride

administration (Hedlund and Gallagher, 1989; Schnitzler et al., 1990; Melton, 1990). It is suggested that fluoride causes resorption to be transferred from the thickened and increasingly metabolically inert trabeculae to the less fluoride loaded cortex (Riggs et al., 1980). Aaron et al. (1991) concluded that doses of fluoride conventionally used in the treatment of established osteoporosis are incapable of restoring abnormal skeletal architecture of idiopathic osteoporosis.

Similarly, several natural and synthetic compounds that inhibit bone resorption interfere with these functions and vice-versa. Hence inhibition of enzymatic degradation with inhibitors of cystein-proteinases or metallo-proteinases (Everts et al., 1992), inhibition of carbonic anhydrase (Waite et al., 1970), sodium pump (Prallet et al., 1988), or proton pump activity (Sundquist et al., 1990) or inhibition of bicarbonate chloride (Hall and Chambers, 1989) or sodium-proton exchange (Hall and Chambers, 1990) all lead to an inhibition of bone resorption.

Roldan and Ferretti, (2000) observed that the therapeutic effects of anti-osteoporotic agents on bone strength cannot be explained only on the basis of simple increases in the bone mass or "areal" mineral density, as if the skeletons were deposits of hard material resulting from a merely quantitative balance between formation and destruction. The understanding of the mechanisms of these effects involves their analysis at different levels of biological complexity

that, rather than to the mineral mass, is related to the mechanical quality and the spatial distribution of the bone material. The bone modeling stimulators enhance the bone mass, chiefly by inducing peritrabecular apposition here and there, with a relatively small improvement (if any) in the bone architectural design. Some of these agents may even deteriorate the mechanical quality of the bone material because of crystal contamination (fluoride) or excessive haversianization (parathyroid hormone [PTH], prostaglandin E2 [PGE2]).

Roldan and Ferretti (2000) concluded that we are facing a conceptual crisis concerning the diagnosis and interpretation of all the bone-weakening diseases and the mechanisms of action and criteria for evaluation of their therapeutic agents. Only a coherent, biochemical interpretation of the skeletal system according to its different levels of biological complexity will allow us to establish the true diagnostic and therapeutic rules focused on the optimum, functional goal, i.e., the optimization of the patient's quality of life.

Materials and Methods:

Experimental Protocol:

Animal : Wister rats

Weight : 150 gms (approx.)

Sex : Male / Female

Anesthesia : Phenobarbitone Sodium (30mg per kg of body weight)

Duration : 70 days

One batch : 16 rats (8 standard & 8 experimental)

Setting of Standard:

Denervation (Sciatic neurectomy): on 1st day in one leg (in anaesthetic condition for each group).

Group 1 : 4 rats

MRI & X-ray (*in-vivo*): on 15th day.

Sacrificed the rat for removal of bone (femur & Tibia).

Group 2 : 4 rats

MRI & X-ray (*in-vivo*): on 30th day.

Sacrificed the rat for removal of bone (femur & Tibia).

Experimental protocol:

Denervation (Sciatic neurectomy): on 1st day in both legs (one for control and other for experimental) in anaesthetic condition for each group.

Exposure of modulated low frequency pulsed signal (16 Hz & 10-volt peak to peak) in experimental legs: 30th day onwards after neurectomy. One leg bounded between 2 electrodes of Bone Stimulator, daily for 2 hours in anesthetic condition during exposure.

Group 3 : 4 rats

MRI & X-ray (*in-vivo*): after 15 days of exposure.

Sacrificed the rat for removal of bone (femur & Tibia).

Group 4 : 4 rats

MRI & X-ray (*in-vivo*): after 30 days of exposure.

Sacrificed the rat for removal of bone (femur & Tibia).

Removed bone was analyzed for Bone mineral density, Bone ash content, cortical thickness and internal structure of bone by Scanning Electron Microscope.

Twenty male Wister rats weighing approximately 150 grams each were taken and divided into 4 groups (5 rats in each group). Each rat of two groups (1st and 2nd groups) underwent only one-sided sciatic neurectomy on first day of seventy days experiment. After the animal had been anesthetized with Phenobarbitone Sodium (30mg per kg of body weight), one hind limb was shaved over the thigh and sterilized. An incision was made on the upper thigh just posterior to the femoral trochanteric region. The sciatic nerve was mobilized within the incision and about 0.5 cm section was excised (having one leg Denerved / Induced and other as Normal / Fresh). The skin incision was closed with sterilized Ethicon thread by using stainless steel needle. Each rat of the rest two groups (3rd and 4th group) underwent sciatic neurectomy in both legs by the same procedure (having one leg as Sham-exposed / Control and other as Exposed / Experimental) on the same date as the first two groups. Powder of antibiotics (Anthrocin 250; equivalent of 250mg of Erythromycin Estolate IP) was given (as needed) through drinking water for easy healing of wound and inhibiting any infection. Each rat was housed singly in $17 \times 18 \times 24$ cm^3 plastic cage and allowed free access to tap water and pelleted commercial diet.

After 15 days of sciatic neurectomy, four rats of first group underwent for MRI and X-ray image *in-vivo*. Then all rats of the first group sacrificed and femur and tibia were resected for further analysis. Same procedure was followed

for the second group of rats (both groups having only one side neurectomy) after 30 days. After 30 days, each rat of the rest two groups got the exposure of low level pulsed signal in one leg daily for two hours. For the exposure, we have developed a bone stimulator that can produce modulated low level pulsed electromagnetic waves of 16 Hz at 10 Volts peak to peak, carried by 10 MHz frequency. Here, 16 Hz is the modulating frequency and 10 MHz is the carrier frequency. Bone stimulator was designed with the following specification:

Carrier Frequency	10.0 MHz
Modulating Frequency	16.0 Hz
Amplitude	10 V (peak to peak)
Wave Shape	Square

Output of stimulator was given to each rat separately by a pair of electrodes in only one leg (Exposed). Other leg was bounded between the same type of electrodes with connection to stimulator, which was not energized (Sham-exposed). Before giving exposure, rats were lightly anesthetized so that they could not disturb the experimental process. MRI and X-ray image of rats of third and fourth group were taken after 15 days and 30 days of exposure respectively. Then rats were sacrificed and femur and tibia were resected for further analysis.

MRI

High resolution imaging of normal body tissues and pathologic processes are possible because of the extraordinary tissue contrast achieved through a variety of techniques. Magnetic resonance imaging (MRI) is a sensitive, noninvasive method for evaluating musculoskeletal disease. MRI uses the principle of the resonance frequency (which is absorption and release of energy by nuclei in an external magnetic field when stimulated by radio frequency energy at a specific frequency) to create body images. The natural abundance and strength with which it interacts with magnetic fields makes hydrogen the most suitable of all nuclei for clinical imaging.

In MRI, the static external magnetic field establishes equilibrium between hydrogen protons aligned parallel and anti-parallel to the external field. RF energy pulses are used to excite protons from the lower-energy parallel alignment into the higher-energy anti-parallel alignment. Upon discontinuation of the radiowaves, the excited protons lose this extra energy through T_1 (longitudinal) relaxation and T_2 (transverse) relaxation mechanisms. These two factors as well as a third factor, the number of hydrogen protons, or proton density determine the intensity of the magnetic resonance signal. These three parameters suggests three basic types of sequences, namely T_1-weighted, T_2-

weighted, and proton density-weighted sequences and resultant images. The spin-echo sequence is the most common sequence utilized in MRI of the musculoskeletal system. The T_1-weighted (short TR / short TE) sequence reveals areas of short T_1 relaxation time as bright signal and areas of long T_1 relaxation time as dark signal. Where, TR and TE are the repetition time and the echo time respectively. Because fat is the most common tissue within the body that has a short T_1 relaxation time, fat is bright on the T_1-weighted image. Similarly, a spin-echo sequence that is T_2-weighted (long TR / long TE) reveals areas of long T_2 relaxation time as high signal intensity and areas of short T_2 relaxation time low signal intensity. Therefore, areas of the body containing excess amounts of free water or liquid such as a bone contusion, cyst, inflammation neoplasia etc. are bright on T_2 - weighted image. Hence, T_2 weighted image of osteoporotic bone due to the presence of body fluids in pores will have more brightness than the normal bone.

MR imaging:

MR images were obtained at 4.7T / 40 cm on ***Bruker 'BIOSPEC'*** (Germany) (Fig: 1a & 1b). MR scanner 69mm volume resonator was used as a receiver and transmitter coil. All the MR imaging experiments were performed in the Department of N. M. R., All India Institute of Medical Sciences. Before MR imaging, rats were anesthetized, to avoid any body movement. Anesthetized rat was kept singly at the center of the magnet and sagittal T_2-weighted spin-echo pilot scans were acquired using 'RARE' pulse sequence. Once the region of interest is located on pilot scans, T_2-weighted multislice spin-echo images were acquired using the same pulse sequence. For this following parameters were used:

Repetition Time (TR) = 2000 ms

Echo Time (TE) = 15 ms

Field of view (FoV) = 6×6 cm^2

Matrix size = 256×256

Size thickness = 2 mm

Acquisition time = 6 min 45 sec

Using 'image processing software', average signal was obtained from the area of interest on transverse images of each rat.

X-ray:

Radiological diagnosis is all important for the diagnosis of bone deformities. It offers the first view of the altered morphology of bone and provides the first information about the possible nature of the lesion. The site of lesion within the affected bone, its intraosseous and extraosseous extent, its form and structural density and many other changes which may contribute decisively to the diagnosis are here made visible. Plain radiograph (X-ray image) is always taken in two perpendicular planes. With this photographic technique the internal structure can be clearly visualized.

The changes in osteoporotic bone are often discovered by using X-ray when well advanced; they are subtle but identifiable. The most striking change is cortical thinning with irregularity and resorption of the endosteal (inner) surfaces. As the bone density decreases, the cortex appears as a thin line that is relatively dense and prominent, producing the typical picture-frame pattern. Spongy bone loses some of trabeculae; those remaining are in line of stress and increase in density and width. For X-ray image we used following parameters:

Power	50 Kv
Current	40 mA
Exposure time	0.2sec

Scanning Electron Microscopy:

In the SEM the electrons are focused to a small beam or probe that is scanned across the upper surface of the specimen. This scan produces a signal that is used to generate an image on a cathode ray viewing screen.

Femur and tibia of one rat of each group were resected and made them free of all soft tissues. Transverse sections of bone of approx.0.5cm thickness were made by sawed edge knife for electron microscopic examination. Then transverse sections of femur and tibia of each leg (normal, osteoporotic, exposed or sham-exposed) separately put into fixative (Glutaraldehyde; provided by electron microscopy facility institution). When cytological details of the bone marrow are required, fixation in Glutaraldehyde (2 ml 25% glutaralaldehyde solution, 3 ml 37% formaldehyde solution, 1.58 g dehydrated calcium acetate, aqua dest ad 100 ml. Minimum fixation time at room temperature is 8 - 24 hrs) is recommended. Then they were washed with phosphate buffer solution and dehydrated till the critical point drying, to keep the surface intact without swelling and shrinking. With silver paint they were mounted on a circular stub. Then the sample was coated with a thin layer (50 - 300A^0) of gold prior to being placed in the microscope to make the sample conductive for taking image, because the image is actually produced by the interaction of the electron beam with this metal coating. SEM photography was performed on 'low vacuum SEM'

Leo 435 VP (Cambridge, England) (Fig: 2). The scale present in the SEM images was used to measure the cortical thickness of bone.

Bone Ash:

Resected femur and tibia of one rat of each group was taken and freed from all soft tissues. Wet weights of those were determined by using electronic balance. Femur and tibia were next placed in individual porcelain crucibles and dried for twenty-four hours in oven at 100 degrees Celsius. After dry weights were determined, femur and tibia were placed in a muffle furnace for twenty-four hours at 800 degrees Celsius and ash weights were determined. These weights were analyzed by comparing the weights of the right femur and tibia with those of the left to determine whether significant osteoporosis had developed in the neurectomized side in the first two groups. Same analysis was done for the third and fourth group to determine whether any differences were present in the exposed and sham-exposed samples.

Results:

All results of MRI have been taken in prone position (Belly down, Back up) of rats. In normal rat, signal intensity of the distal femur in T2-weighted MR spin-echo images of transverse as well as in coronal sections of both legs is quite similar (Fig: 3a & 3b). Whereas the signal intensity (brightness) of the image increases in the osteoporotic left leg after one month of neurectomy in comparison to the normal right leg in group two rats (Fig: 4a & 4b; both images are of the same rat but in different image). After one month of inducing osteoporosis, exposure of field for one month in right leg of group four rats shows reduction in intensity of signal in comparison to sham-exposed left leg (fig: 5a & 5b). Rats of group one and group three did not show any marked difference in the interested region. On gray scale of area of interest (extreme end of femur), the T2-weighted MR spin-echo images revealed the lowest signal intensity in the normal bone and the highest signal intensity from the sham exposed bone in the order of,

Normal < One month osteporotic < Exposed < Sham exposed. (Table: 1)

In SEM images, transverse sections of femur and tibia of normal rat reveal smooth surface (Fig: 6a, 6b, 6c and 6d). In the osteoporotic femur and tibia, honeycomb structure appears in the marrow and calcaneous part in rats of

group one and two (Fig: 7a, 7b, 7c and 7d). Whereas after 15 days of exposure, honeycomb structure started to disappear in comparison to sham-exposed samples in the third group of rats (Fig: 8a, 8b, 9a and 9b). After 30 days of sham-exposure in one-month osteoporotic leg, large holes with sharp edges appear in calcaneous bone (Fig: 10a and 10b). Whereas in exposed bone, holes start reducing in dimension by the deposition of minerals (Fig: 11a and 11b).

X-ray image of normal rat shows the normal intensity of brightness in both femur and tibia (Fig: 12). After one month of neurectomy, left bone shows cortical thinning and less brightness in bone marrow part in comparison to right (Fig: 13). One month exposure of low level electric field in one month induced osteoporotic right leg reduced the cortical thinning as well as increased the brightness of bone marrow part compared to left one (Fig: 14 and Table: 2). All X-ray images have been developed for the proper analysis.

Table: 1. MR Image signal intensity of particular area of interest (distal femur) on gray scale.

	Intensity of particular area on gray scale
Normal	59.1±15.6
After one month of Denervation	82.6±13.3
After one month of Exposure	102.2±16.4
After one month of Sham-exposed	128.6±11.9

Table: 2. Cortical thickness of tibia at various period of experiment.

	Cortical Thickness (in μm)
Normal	461.67±53.93
After 15 days of Denervation	371.67±30.14
After 30 days of Denervation	296.00±190.90
After 15 days of Exposure	445.00±57.66
After 30 days of Exposure	510.00±155.56

Table: 3. Bone mineral content in femur and tibia at different period of Experiment.

	Femur			Tibia		
	Bone weight (in mg)	Ash weight (in mg)	% mineral	Bone weight (in mg)	Ash weight (in mg)	% mineral
Normal	52	12	23.1	40	11	27.5
½ month Denerved	49	9	18.4	36	8	22.2
Normal	68	19	27.4	53	16	30.1
1 month Denerved	62	12	19.3	39	9	23.1
1 month Exposed	67	17	25.4	46	12	26.1
1 month Sh-am exposed	67	14	20.9	47	10	21.3

Fig: 1a. MRI instrument where rat is kept singly for imaging.

Fig: 1b. Screen of MRI instrument.


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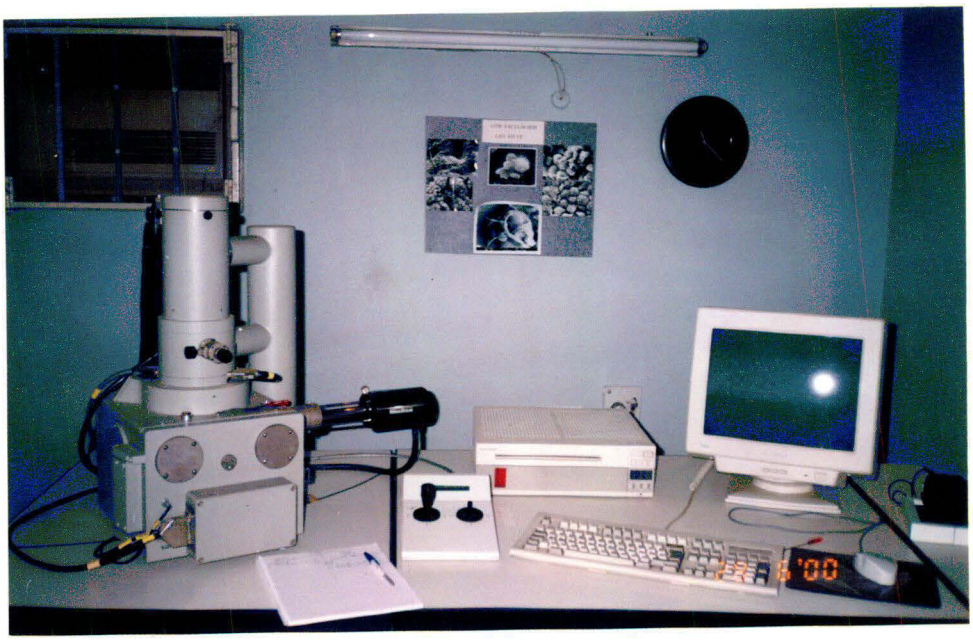
Fig: 2a. Scanning Electron Microscope.

Fig: 2b. Exposure of rat by bone stimulator.



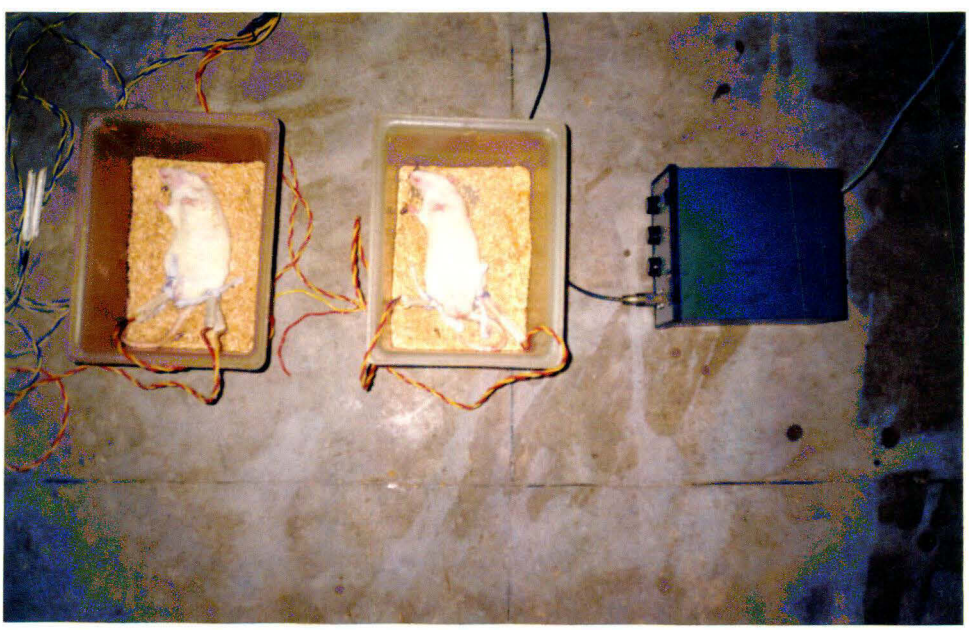
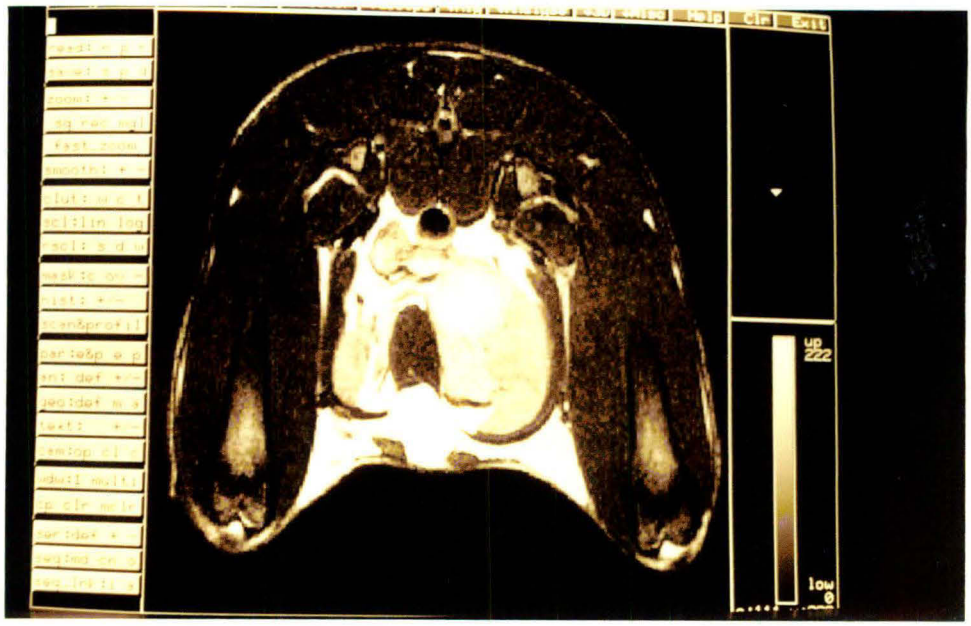


Fig: 3a. Transverse image of normal rat.

Fig: 3b. Coronal image of normal rat.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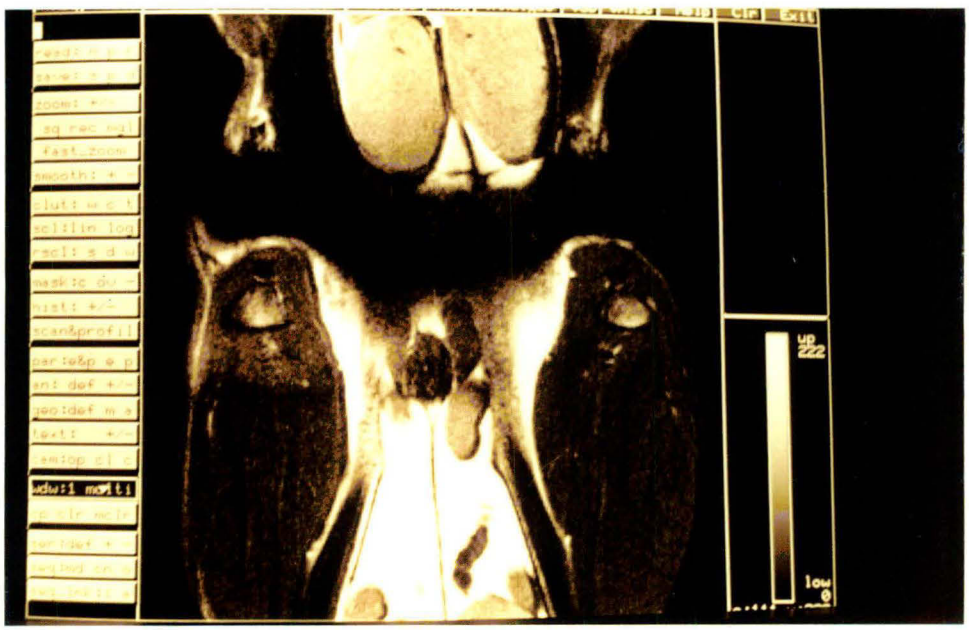
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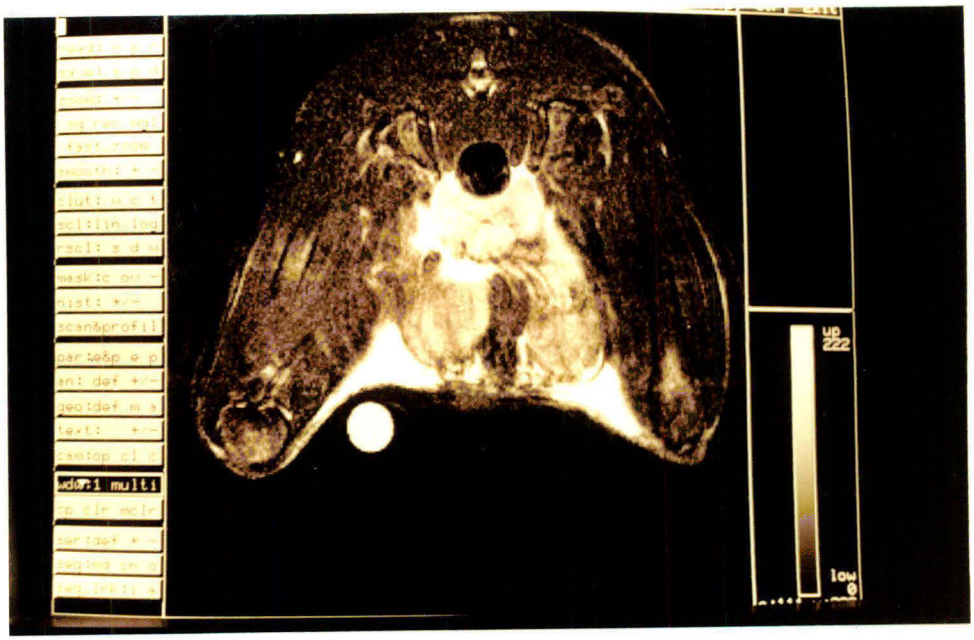
Fig: 4a. Transverse image of rat after 30 days of Denervation (Neurectomy).
(Right leg normal)

Fig: 4b. Transverse image of rat after 30 days of Denervation (Neurectomy)
(Left leg denerved)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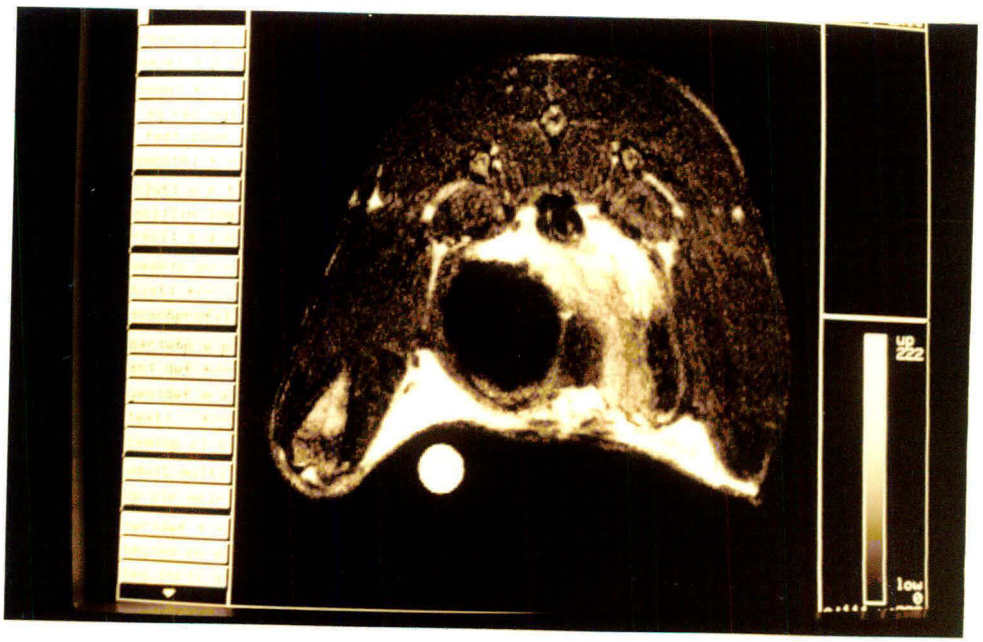
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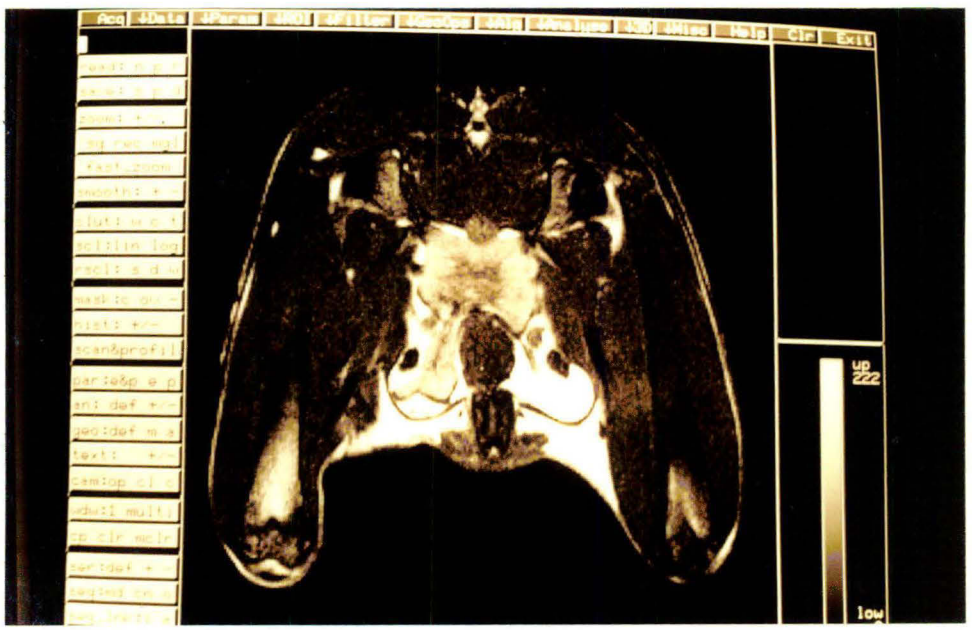
Fig: 5a. Transverse image rat after 30 days of Exposure. Left leg is Sham exposed, whereas right leg is exposed.

Fig: 5b. Coronal image of rat after 30 days of Exposure. Left leg is sham exposed whereas right leg is exposed.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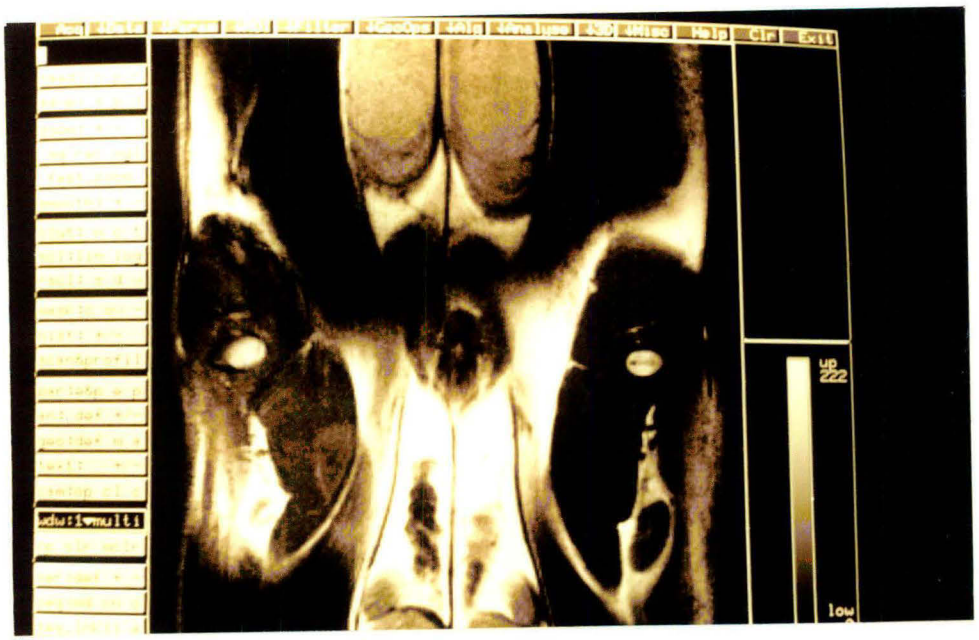
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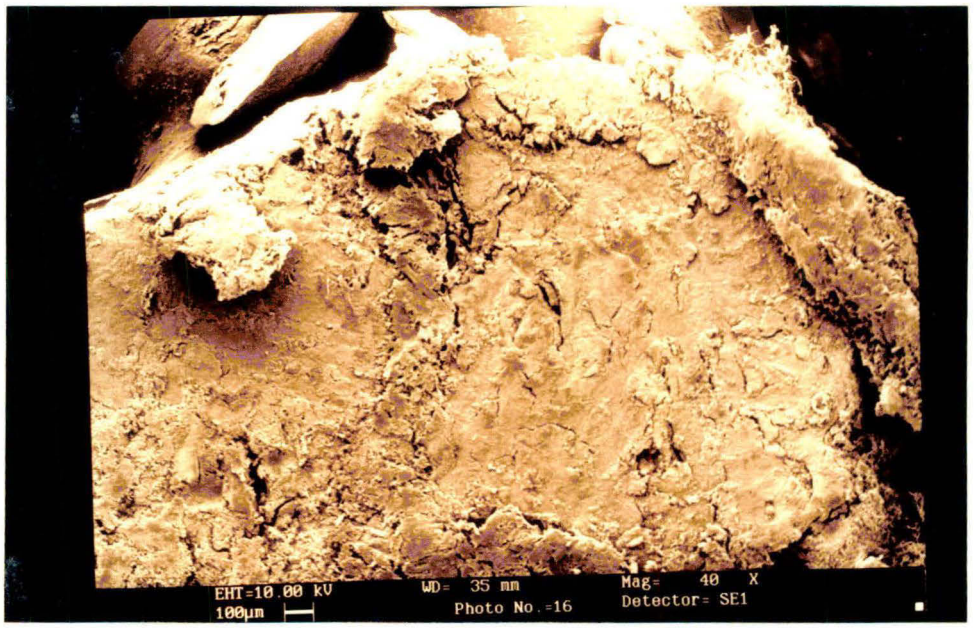
Fig: 6a. T. S. of distal femur of normal rat showing calcaneous part.

Fig: 6b. T. S. of distal femur of normal rat showing trabecular part.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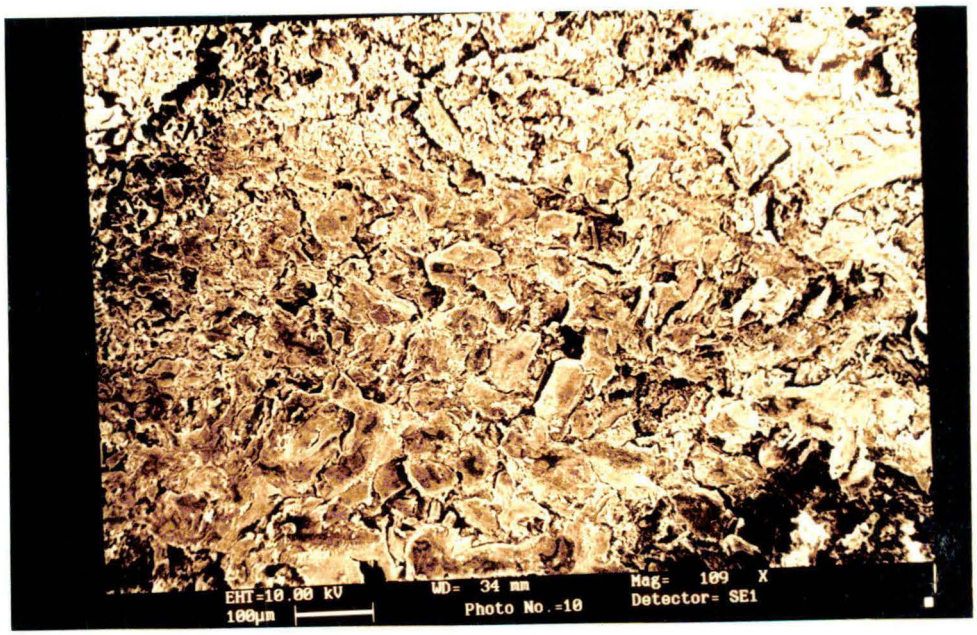
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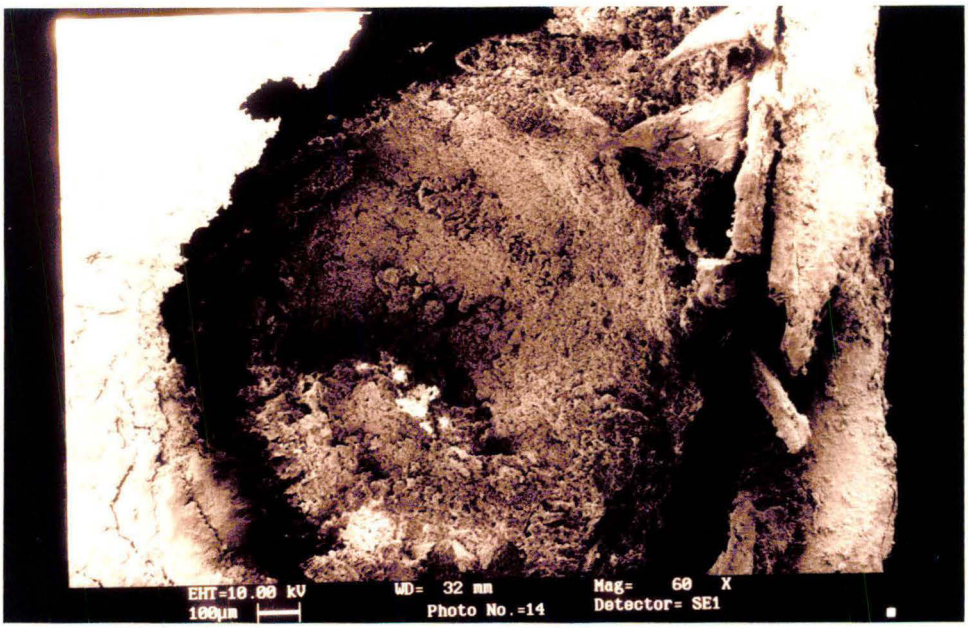
Fig: 6c. T. S. of tibia of normal rat showing marrow part.

Fig: 6d. Side surface of tibia head in normal rat.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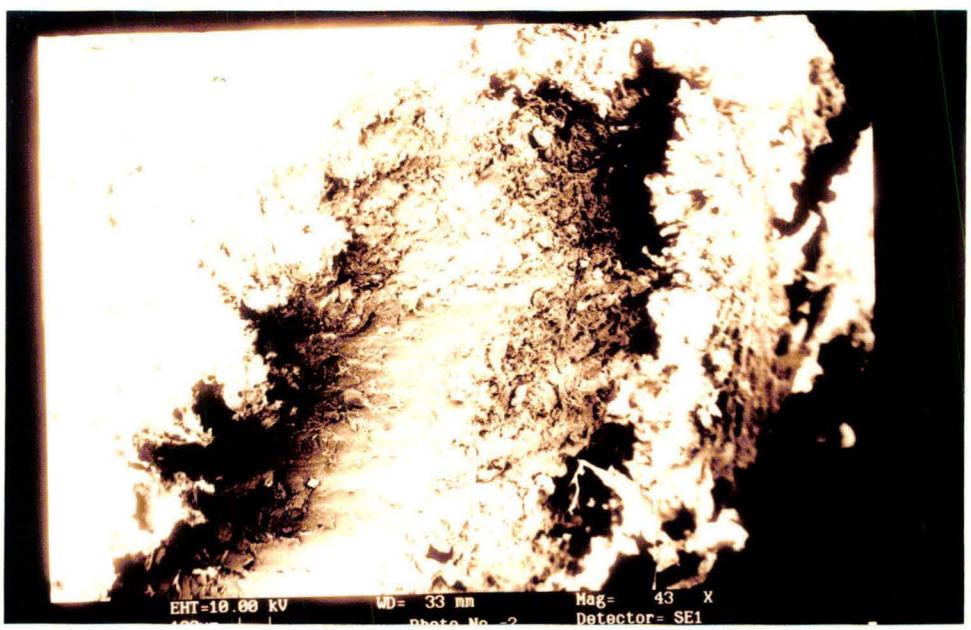
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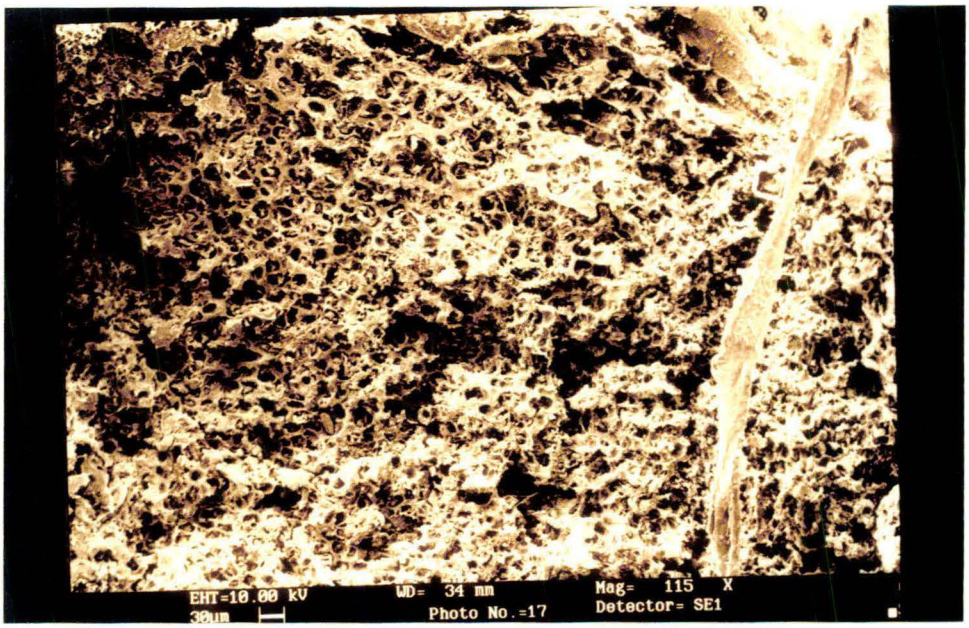
Fig: 7a. T. S. of calcaneous part of distal femur after one month of denervation.

Fig: 7b. T. S. of tibia after one month of denervation (showing marrow part).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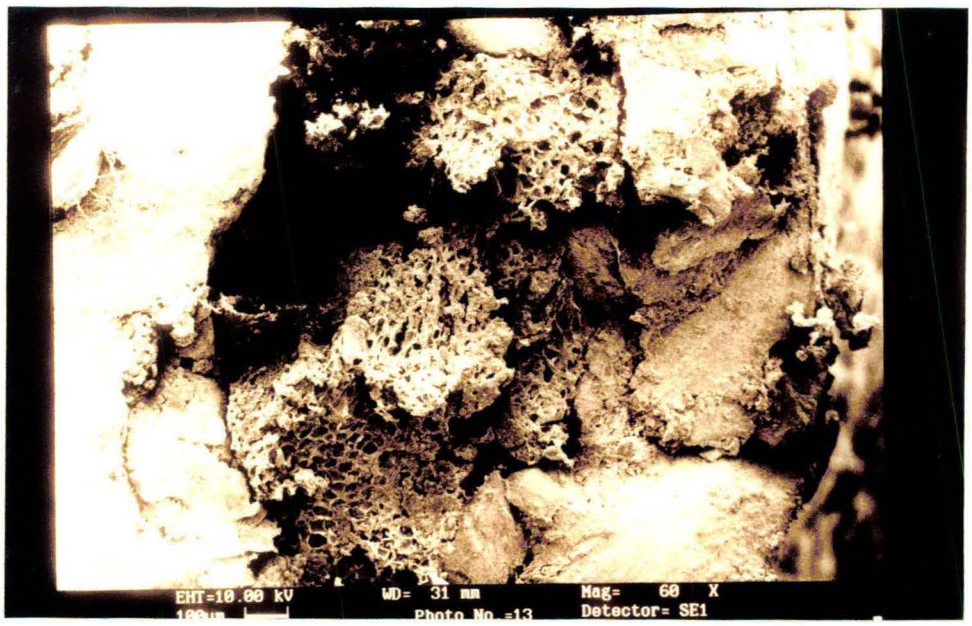
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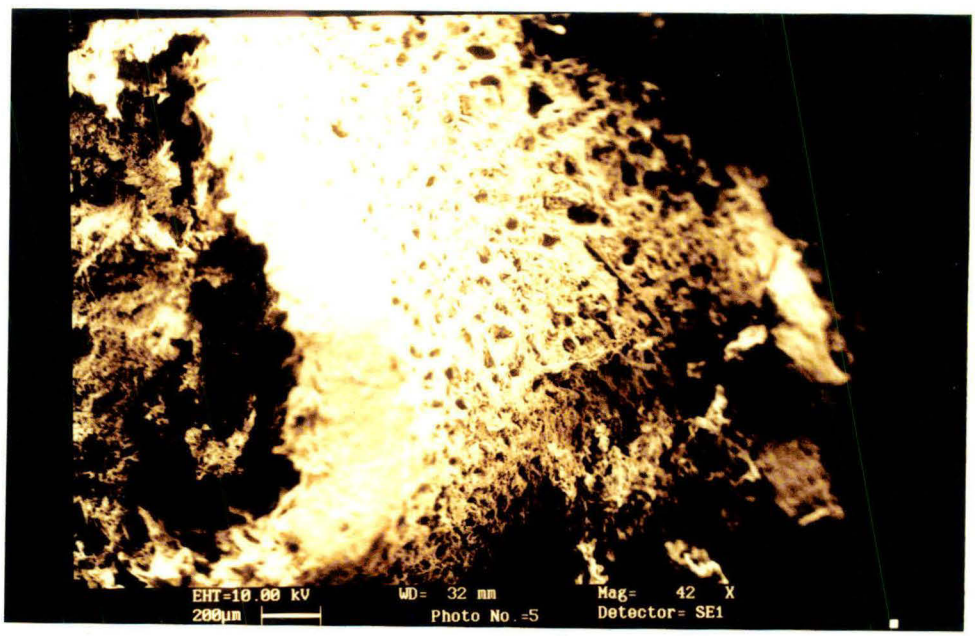
Fig: 7c. Side surface of tibia head after 15 days of denervation.

Fig: 7d. Side surface of tibia head after 30 days of denervation.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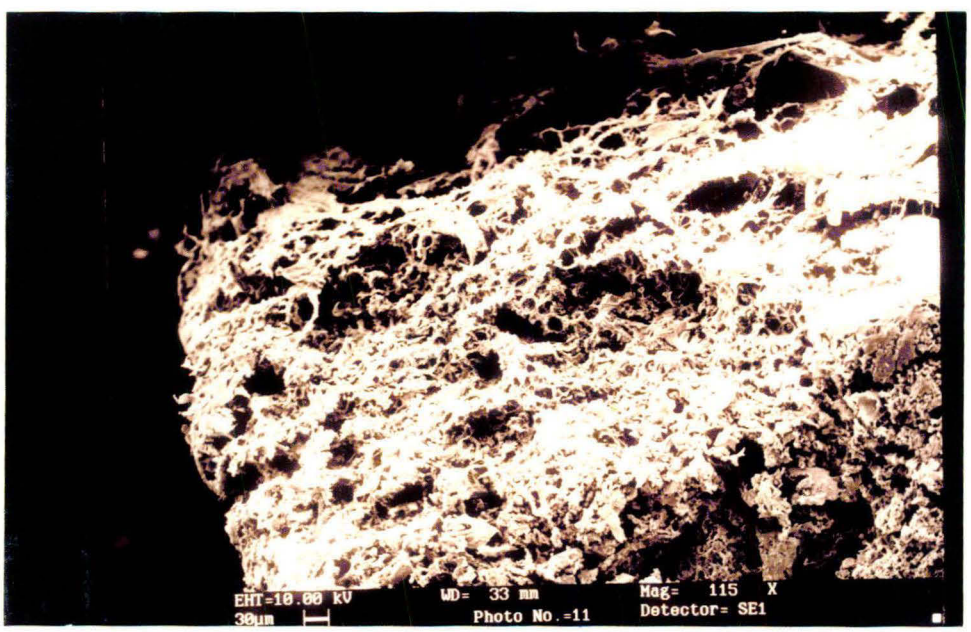
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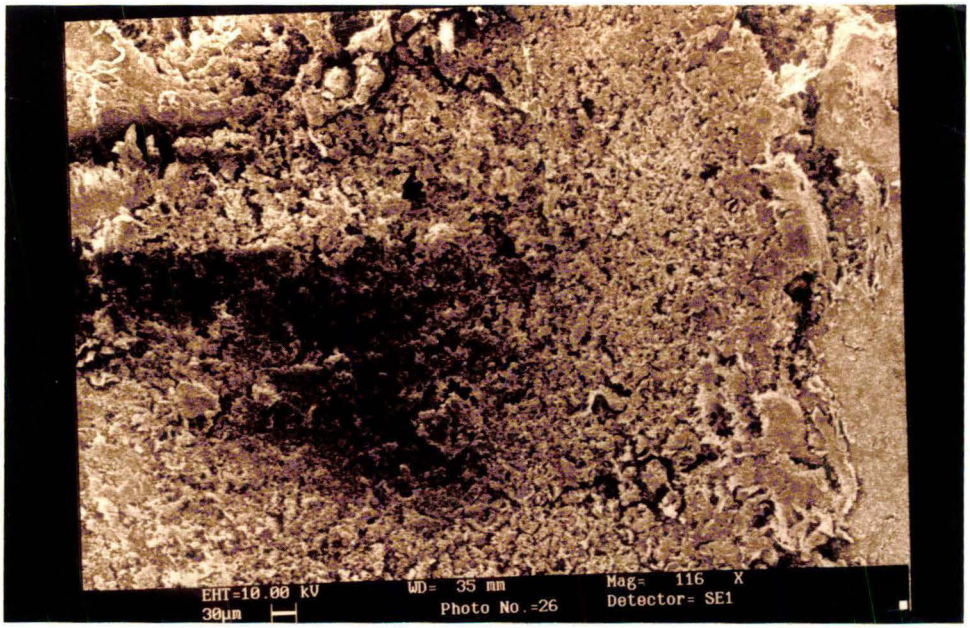
Fig: 8a.T. S. of calcaneous part of distal femur after 15 days of Exposure.

Fig: 8b. T. S. of tibia after 15 days of Exposure showing marrow part.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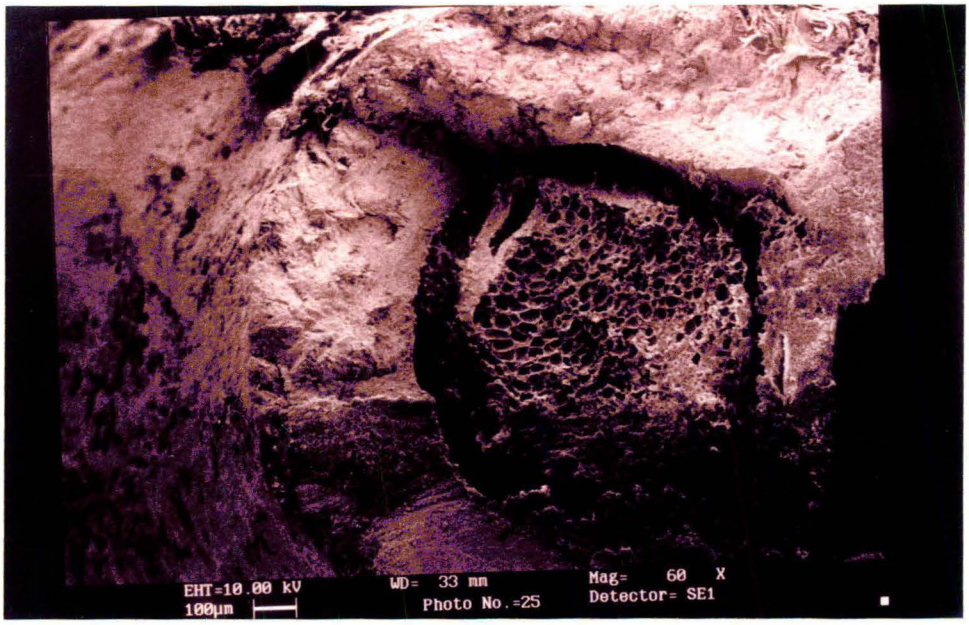
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30μm
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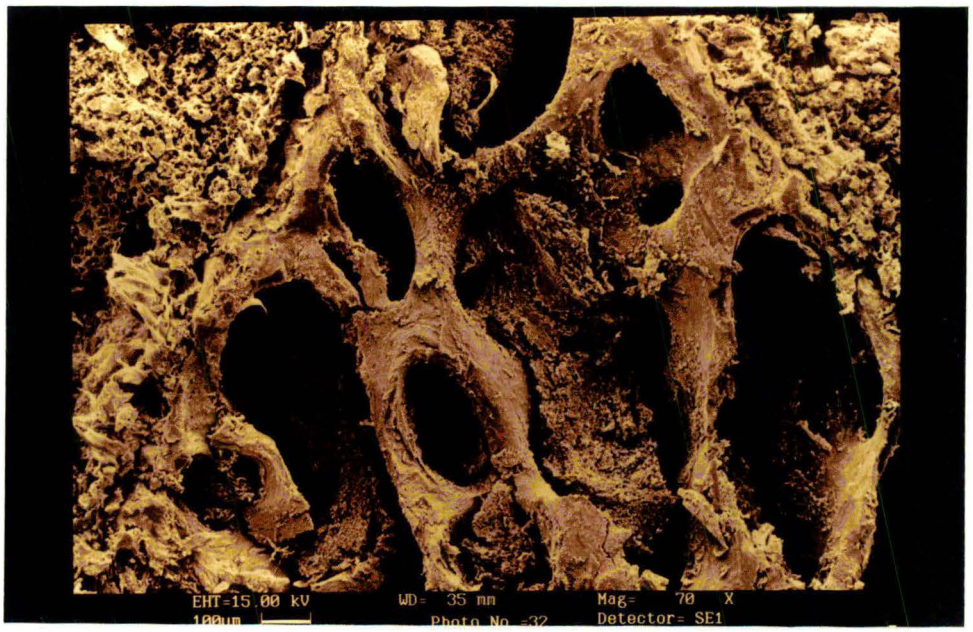
WD= 32 mm
Photo No.=25
Defocus= 251
Mag= 121 X

100μm
EHT=10.00 kV
WD= 36 mm
Photo No.=55
Defocus= 251
Mag= 181 X

Fig: 10a. T. S. of distal femur after 30 days of Sham exposure.

Fig: 10b. T. S. of distal femur after 30 days of Sham exposure.


EHT=15.00 kV
WD= 35 mm
Mag= 70 X
100µm
Photo No.=32
Detector= SE1

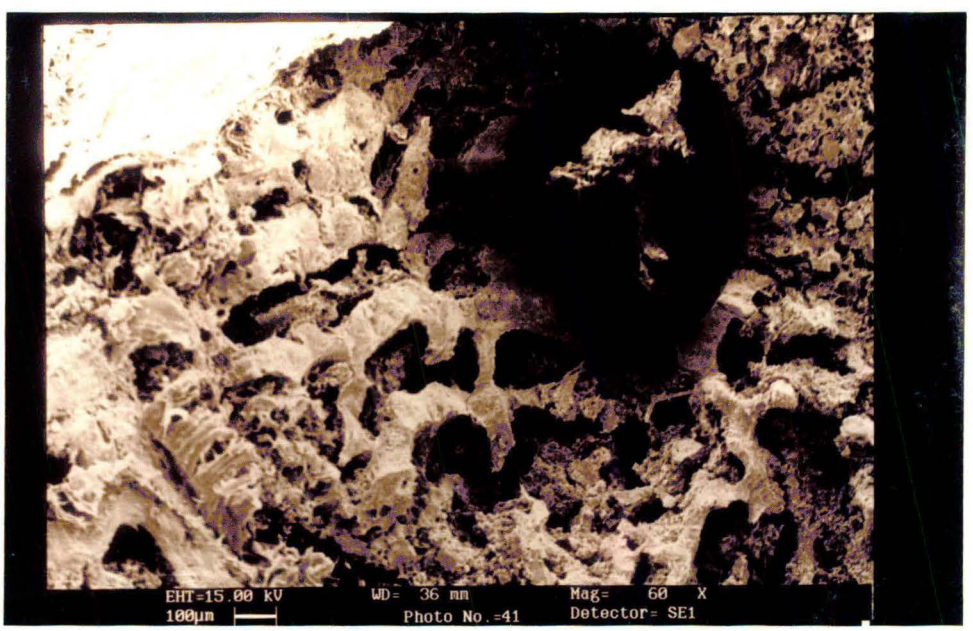
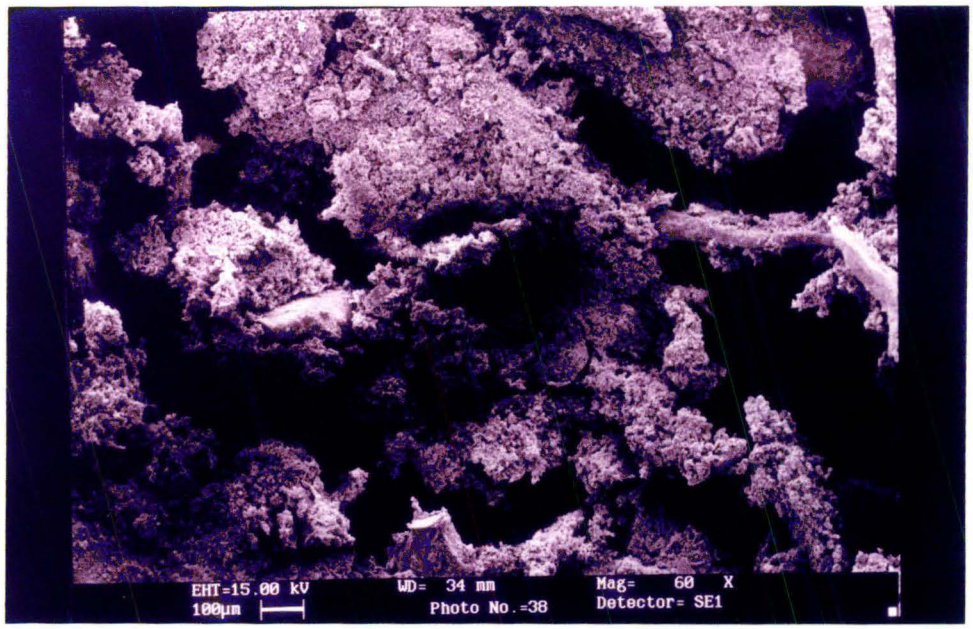

EHT=15.00 kV
WD= 36 mm
Mag= 60 X
100µm
Photo No.=41
Detector= SE1

Fig: 11a. T. S. of distal femur after 30 days of Exposure.

Fig: 11b. T. S. of distal femur after 30 days of Exposure.


EHT=15.00 kV
100µm
WD= 34 mm
Photo No.=38
Mag= 60 X
Detector= SE1

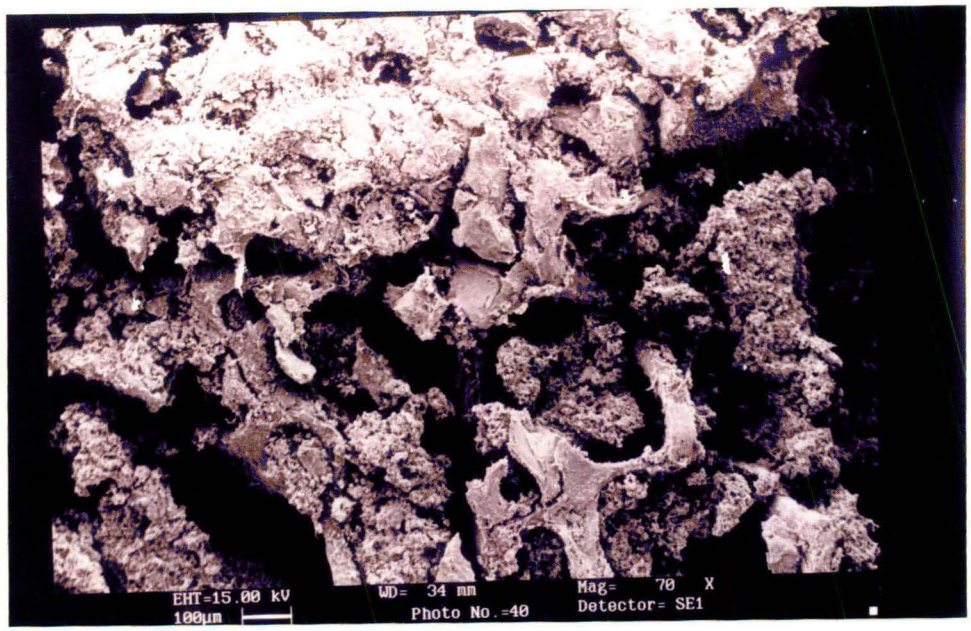
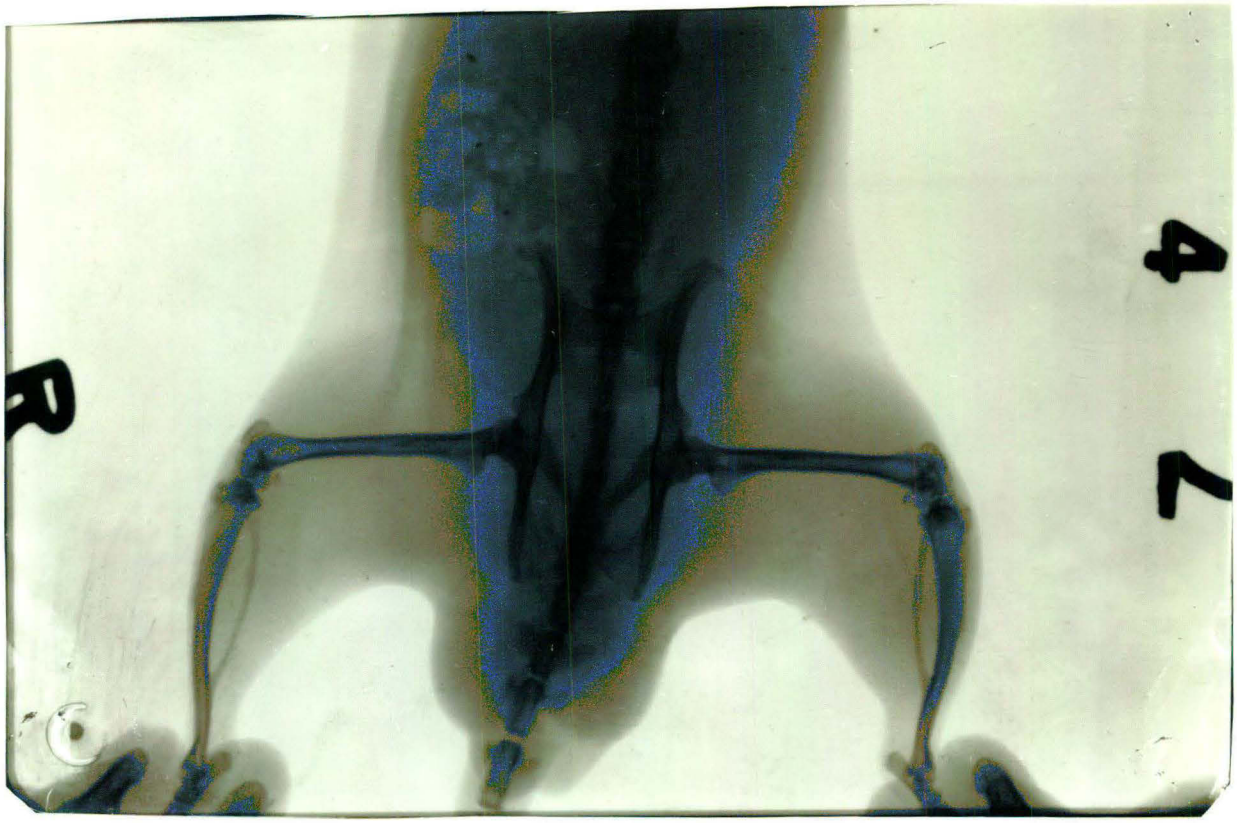

EHT=15.00 kV
100µm
WD= 34 mm
Photo No.=40
Mag= 70 X
Detector= SE1

Fig: 12. X-ray image of normal rat.

Fig: 13. X-ray image of rat after one month of denervation.
Left leg is denerved.


R
4
7

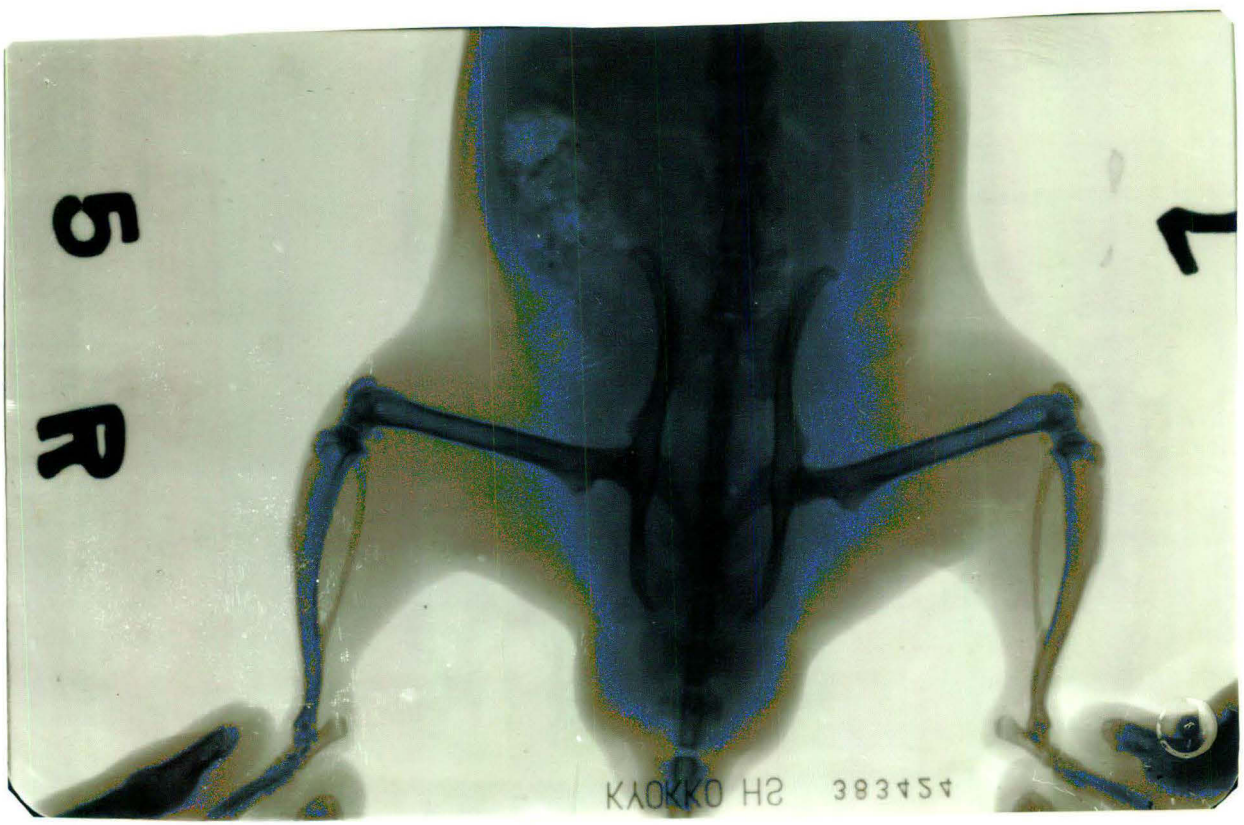

5
R
7

Fig: 14. X-ray image of rat after 30 days of Exposure.
Right leg is exposed.

3
R
KAKKO HS

Discussion:

The basic idea emerges as, in bone, the relationship between mechanical and electrical inputs is hierarchical, with mechanical forces being the more fundamental. Mechanical forces influence bone cell activity by first generating endogenous electrical fields in the matrix (i.e., stress generated potentials) and also an exogenous electrical stimulus to stimulate bone formation (Backer and Murray, 1970; Gross and Williams (1982). Because the known nonmechanical controlling factors for bone remodelling and repair (e.g., hormones, growth factors, vitamin D, calcium) coupled chemically via strong forces of submolecular extent (Mundy, 1995), it seems unlikely that mechanical strain would have evolved with a unique, electromagnetically dependent, biological interaction mechanism.

The bone contains sensor cells that monitor mechanical strain and activate corrective biological processes. Osteocytes, distributed throughout the bone matrix, are bone mechanosensing cells (Marotti et al. 1990). Osteocytes produce a signal proportional to mechanical loading by sensing strain on bone surfaces through stretch-activated ion channels (Duncan and Misler, 1989), flow of interstitial fluid (Cowin et al. 1995), electrical potentials (Harrigan and Hamilton, 1993), or some other phenomenon. These osteocytes are ideally

located for this function, and apparently communicate with one another, as well as with osteoblasts and bone lining cells, through dendritic processes and gap junctions, forming a functional syncytium (Doty, 1981). Cell to cell communication of electrical signals and small molecules through gap junctions has been demonstrated in osteoblasts (Jeansonne et al, 1979; Schirrmacher et al, 1992; Donahue et al, 1995). Similar gap junctions in osteocytes (Gray et al, 1996) participate in such communication with osteoblasts, and bone lining cells as well.

Martin (2000) assumed that when the osteocyte signal becomes sufficiently great it ceases to inhibit bone lining cells from remodeling and causes them to revert to a bone forming phenotype. This could be responsible for the observations of lamellar bone modeling on endosteal surfaces (Turner et al, 1998), and vertebral trabecular surfaces (Chow et al, 1998) under increased loading. He proposed that a single kind of signal transmitted through bone's cellular syncytium of osteocytes, osteoblasts, and lining cells controls remodeling in response to extremes of mechanical loading and such exigencies as osteocyte death and hormonal fluctuations. His theory recognized that these three kinds of bone cells are inexorably linked not only by gap junctions, but through a common differentiation pathway as well. Osteoblasts change into osteocytes and bone lining cells seamlessly, and none of the three can realize

their full function in remodeling without the other two. Osteocytes use the common signal to guide osteoblasts into the bone matrix, serves to gauge its burden of mechanical stress and damage, and signals bone lining cells when it is time to remove and replace the tissue.

Images of various parts of transverse section of femur and tibia shows the clear differences in normal, osteoporotic and exposed bone. Smooth surface of normal bone in transverse section (Fig: 6a and 6c) indicates the compact deposition of minerals in bone. Denervation induces the osteoclastic activities in trabecular as well as endosteal part of bone and makes the bone thin and porous (Fig: 7a). With the increasing time period, more osteoclastic activities encreased the porosity in bone (Fig: 7c and 7d). With the exposure of low level pulsed electromagnetic field the amount of porosity started to reduce (Fig: 8a, 8b and 9b) and clearly showed the positive effect of the ELF exposure. Reduced signal intensity in the area of particular interest (distal femur) in MR images with the support of electron microscopy images, X-ray images, cortical thickness and bone ash content gives the valuable information about the reversal of induced osteoporosis in rat bone by this non-invasive method. After one month of exposure, deposition of minerals near the sharp edges of pores, clearly shown in T. S. of exposed bone (Fig: 11a and 11b). Cortical thickness in femur as well as in tibia is reduced in the X-ray images of osteoporotic bone (Fig: 13), and it has

been slightly increased after one month of exposure (Fig: 14), indicating the osteoporotic process in endosteal part is also reversed (Table: 2). Decreased percentage of minerals in bone ash of osteoporotic bone in comparison to normal and increased percentage of minerals in bone ash of exposed bone in comparison to sham-exposed bone also suggest about the positive effect of ELF (Table: 3).

Low frequency, low energy pulsed electromagnetic fields (PEMFs) are used to stimulate healing of ununited bone fractures in human. Experimental and clinical research studies have been shown the positive effects of PEMFs on endochondral bone formation and on osteogenesis. It is shown that extremely low frequency EMF exposure can alter Ca^{2+} transport, probably through Ca^{2+} channel without having any such effect on non-activated cells (Walkeczek and Liburdy, 1990). The temporary application of a 60 Hz sinusoidal E-fields causes some dynamic changes in cell membrane components and/or within the vicinity of cellular membrane, reflecting in reduced or induced Ca^{2+} influx respectively through ATP or histamine induced ion channels. Even though the observed ELF effects have been temporary, it is possible that chronic exposure of low intensity ELFs could have long lasting effects on cell physiology, through changes of Ca^{2+} distribution within the cells (Lyle et al., 1991).

Harrigan and Hamilton (1993) and Zhang et al (1997) suggest that the mechanical loading is converted to an electrical signal that can be transmitted intracellularly to the bone lining cells, creating intracellular or transmembrane potential changes in the osteoblasts. Mcleod and Rubin (1992) demonstrate that a simple, low power sinusoidal field induced at 15 Hz is the most osteogenic of any induced field they studied. As osteoporosis is characterized by the loss of mineralized bone, which results in structural fracture. The probability of fracture is closely related to a person's effective bone mass, a modality that could prevent or retard the loss of bone might provide a substantial reduction in the incidence of skeletal morbidity. As a non-invasive technique the exposure of PEMFs at particular frequency (i.e. 16Hz) into the bone and surrounding tissues can substantially and beneficially influence the behavior of bone cell population that are responsible for the bone resorbing and bone forming.

All the facts described above clearly suggest about the reversal of the osteoporotic process by the exposure of ELF. Although, it is clear that mineralization occurs in the osteoporotic bone and bone mass increases after the PEMFs exposure, it is not as much as compact as the original structure (Fig: 11a & 11b in comparison to 6a & 6b). It seems that the deposition of minerals occurs like the filling of a container with sand. Increase in bone mass may not

necessarily followed by an improvement in bone quality. How much this mineralization gives the strength to the bone is still to be evaluated.

Later on, this experiment may be shifted to the exposure by Laser Irradiation rather than by Bone Stimulator. Very Low Power Laser Irradiation (1 mili-watt approx.) guided by Optical Fiber at the particular part of bone can significantly alter the metabolic activities of bone.

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