QUANTITATIVE ANALYSIS OF BONE LOSS IN WISTAR RAT EXPOSED TO SIMULATED MICROGRAVITY

Dissertation submitted in partial fulfillment of the requirement for the degree of

MASTER OF PHILOSOPHY

BY

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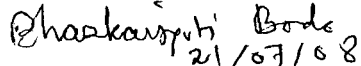## JAWAHARLAL NEHRU UNIVERSITY

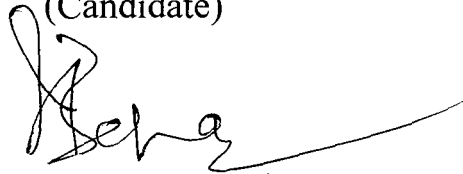**NEW DELHI– 110067**

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Dedicated to my parents and Teachers

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CERTIFICATE

The research work embodied in this thesis entitled *QUANTITATIVE ANNALYSIS OF THE BONE LOSS IN WISTAR RAT EXPOSED TO SIMULATED MICROGRAVITY* 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 degree or diploma of any University .

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Contents:

LIST OF ABBREVATION

AAS	Atomic Absorption Spectroscopy
ALP	Alkaline Phosphatase
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BOC	Bone Organic Content
cAMP	cyclic-Adinosine Mono Phosphate
DMSO	Dimethyl Sulphoxide
EDTA	Ethelene Diamine Tetraacetic Acid
ELF	Extremely Low Frequency
EMFs	Electromagnetic Fields
ER	Etrogen Receptors
FAAS	Flame Atomic Absorption Spectrometer
HLS	Hindlimb Suspension
HE	Hematoxylin and Eosin
HAP	Hydroxyapatite nanoparticle
IGF	Insulin Like Growth Factor
IP	Intraperitoneal
OB	Osteoblast
OC	Increased Osteoclast
OPG	Osteoproteg
PTH	Parathyroid Hormone
RANK	Receptor Activator of NF-kB
RANKL	Receptor Activator of NK-kB Ligand
SC	Stromal Cell
SCGE	Single Cell Gell Electrophorosis
SEM	Scanning Electron Microscopy
TGF-β	Transforming Growth Factor--β
VMR	Vanadate – Molybdate Reagent

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CHAPTER-1
INTRODUCTION

INTRODUCTION

Exploration is an important survival strategy in evolution. The migration of expansive species depends on exploring their immediate or distant surroundings for new food sources or safe habitats ;it may also come as a result of population pressures or environmental changes.human species has added another reason for exploration ,namely curiosity.

Scientist have long worried about the adverse effects of space flight; indeed when Yuri Gagarin made his historic flight in 1961 on Vostok I, it was limited to a single orbit around the earth amidst fears that prolonged exposure to the space environment might prove deadly . While these fears turned out to be exaggerated, it is now evident that the space environment does produce several effects on both man and microbes which are of concern to space travelers.

The unique stresses encountered in space include physical factors, such as exposure to markedly diminished gravity and unusually strong radiation, as well as psychological stress caused by isolation and confinement to a restricted area . Sleep disruption, lack of appetite and consequent inadequate nutrition pose additional problems. While all aspects are important, much interest has recently centered on the effects of decreased gravity.Since life on this planet evolved with gravity as a constant feature , there is curiosity about how earthly life copes with the entirely unprecedented experience of reduced gravity

1.1Microgravity: :. The presence of Earth creates a gravitational field that acts to attract objects with a force inversel yproportional to the square of the distance between the center of the object and the center of Earth. The acceleration of an object acted upon only by Earth's gravity at theEarth's surface is refered as one g or one Earth gravity. This acceleration isapproximately 9.8 meters per second squared (m/s2).The acceleration experienced by an object in a microgravity environment would be the million (10^{-6})of the experience of the earth's surface. Therefore, a microgravity environment is one in which the apparent weight of a system is small compared to its actual weight due to gravity. Researchers can create microgravity conditions indifferent ways. Because gravitational pull diminisheswith distance, one way to create a microgravity environment (following the quantitative definition)is to travel away from Earth. To reach a pointwhere Earth's gravitational pull is reduced toonemillionth cf that at the surface, you wouldhave to travel into space a distance of 6.37million kilometers from Earth . This approach is evident for automated spacecraft,   However, freefall can be used to create a microgravity environment consistent with our primary definition of microgravity.

1.2 Possible mechanism of Bone Loss in Space Flight:

The human skeleton has evolved in the environment of Earth's gravity. This gravitational force plays an important rule in the development of our skeleton system. Therefore, the removal of gravity during long-duration space flight induces several physical responses such as a fluid shifts from the lower extremities to upper body, repositioning of certain organs, musculoskeletal unloading, and a lack of stimulus to the vestibular system . These adjustments can lead to physiological adaptations that are detrimental to health such as decreases in mineral density in weight bearing bones and

orthostatic intolerance , loss of homeostasis in the skeleton, which adapts to the new environment by shedding calcium at a rate that is almost 10 times greater than that in a postmenopausal woman [Iki et al 1996; Sirola et al., 2003].This adaptation to microgravity renders the skeleton "at risk" for fracture, increases the risk of renal stones

It is not well known which mechanism or mechanisms contribute to bone loss and it makes very difficult to determine a treatment that could cure this decrease.The two main physical factors responsible for the physiological changes in bone metabolism are (1) Weightlessness, which leads the muscles to work at much lower load, resulting in smaller strains in bone; and (2) redistribution of tissue fluid pressure, as in normal gravity there is a gradient of tissue fluid pressures, whereas in microgravity this gradient disappears. For example , in a person standing upright under normal (sea level) Earth gravity, the mean blood pressure at the head is 70 mmHg, 100 mmHg at the heart, and 200 mmHg at the feet. Under microgravity condition the blood pressure decreased to 100 mmHg throughout the whole body, and there is cephalic fluid shift (Hargens and Watenpaugh, 1996).The redistribution of tissue pressures does have significant impact on the function of the cardiovascular system, but the most easily visible manifestation of this is the swelling of the face, and the thinning of the legs within a short time of exposure to microgravity.

Using the hindlimb-limb suspension (HLS) rat to model spaceflight, it has been observed that skeletal unloading and cephalic fluid shifts altered bone blood flow. It has been observed that perfusion is diminished in the hind-limb bones and increase in the skeletal structure of forelimbs and head. The decline in blood flow to the hindlimb bones appears to coincide with a diminished mineral apposition rate, density, and mass of both cortical and cancellous bone observed in HLS rats . Correspondingly, the acute increase in blood flow to forelimb, shoulder, and head bones appears to coincide with some reports of increased bone mass in HLS rats . The alterations in blood flow alter the bone interstitial fluid flow and it leads to modulate bone remodeling . Interstitial fluid flows radially through cortical bone, driven by a transmural pressure gradient between the vasculature of the endosteal surface and the periosteal lymphatics . At normal g, Mechanical loading of the skeletal system causes fluid flow through cancellous bone and the lacuna

canalicular network of cortical bone, exacerbating flow induced shear stress imposed on surface bone cells . The shear stresses generated by bone interstitial fluid flow appear to be of similar magnitude to those occurring at the blood-vascular endothelium interface . Bone cells respond to fluid shear forces in a manner similar to vascular endothelial cells, by generating autocrine/paracrine signals that modulate remodeling activity For example, cultured osteoblasts increase production of nitric oxide (NO) and PGE_2 when exposed to elevated flow-induced shear stress, but mechanical strain does not elicit a similar release . NO has been shown to be an osteoblast mitogen with an inhibitory effect on osteoclast bone resorption . PGE_2 stimulates bone formation and attenuates bone loss with immobilization in vivo , presumably via stimulation of osteoblast mitosis, reduction of osteoclast number , and inhibition of osteoclast activity . However, the maintenance of a high interstitial fluid pressure, which serves to offset the chronically elevated arterial perfusion pressure may provide a chronic stimulus to increase bone formation through a NO (21, 42) or PGE_2 signaling mechanism.

HINDLIMB UNLOADING

↓ Perfusion Pressure
↓ Metabolic Rate (?)

↓ Bone Blood Flow

↓ Capillary & Sinusoid Filtration   ↓ Vascular Shear Stress

↓ Interstitial Fluid Pressure

↓ Interstitial Transcortical Fluid Flow
↓ Interstitial Trabecular Fluid Flow

↓ Interstitial Shear Stress

↓ Osteoblast PGE₂   ↓ Osteoblast NO   ↓ ED-NO   ↓ ED-PGI₂
                         ↓ Osteocyte NO

↓ Osteoblastic Replication   ↓ Osteoclastic Inhibition   ↓ Osteoclastic Inhibition
and Differentiation   ↓ Osteoblastic Proliferation

↓ Bone Formation   ↑ Bone Loss   ↑ Bone Loss
                        ↓ Bone Formation

Fig 1: Hypothetical sequence of events linking the unloading of hindlimb bones and the reduction in skeletal blood flow to a remodeling imbalance that leads to a net loss of bone. Two possible mechanisms are illustrated, that mediated through a diminished interstitial shear stress and that mediated through a reduced vascular shear stress. PGE₂; prostaglandinE₂; NO, nitric oxide; ED-NO, endothelium derived nitric oxide; ED-PGI₂, endothelium-derived prostacyclin.

1.3.1 Osteoporosis

Osteoporosis, is a disease that causes bones to become fragile and brittle and very susceptible to fractures. In osteoporosis the bone mineral density (BMD) is decreased, bone microarchitecture is disrupted, and the amount and variety of non-collagenous

proteins in bone is changed.. It is likely to be caused by complex interactions among local and systemic regulators of bone cell function. The heterogeneity of osteoporosis may be due not only to differences in the production of systemic and local regulators, but also to changes in receptors, signal transduction mechanisms, nuclear transcription factors, and enzymes that produce or inactivate local regulators. Within the last decade,the study and identification of the regulatory mechanisms in bone established that osteoporosis is linked to genetic. Since the first human osteoporosis study indicated an association among bone mass, fragility, and polymorphisms in the *vitamin D receptor* (*VDR*) gene, more than 30candidate genes have been reported that might influence skeletal mass and fragility .

Osteoporosis can be considered a consequence of multiple genetic, physical, hormonal and nutritional factors. Typical symptoms of an osteoporotic stage are increased bone resorption in proportion to bone formation, reduced bone mineral density, decreased trabecular bone volume, and as a consequence, impaired mechanical properties of bone resulting in an increased risk of bone fractures. Osteoporosis affects both sexes along with aging. However, in women, estrogen deficiency following the loss of ovarian function in menopause or after surgical ovariectomy, results in the most profound alterations in the skeletal metabolism.

1.3.2Types of Osteoporosis:

Osteoporosis can be classified in various ways based on diagnostic categories, etiology, or stage to help clinicians manage their patients. These classifications include the WHO (world health organization) classification that is *Primary osteoporosis* and *Secondary osteoporosis*. Primary osteoporosis is simply the form seen in older persons and women past menopause in which bone loss is accelerated over that predicted for age and sex. Secondary osteoporosis results from a variety of identifiable conditions.

1.3.3 Primary Osteoporosis

There are two kinds of primary osteoporosis: type I osteoporosis and type II osteoporosis. The determining factor for the actual existence of osteoporosis, whether type I or type II, is the amount of calcium left in the skeleton and whether it places a person at risk for

fracture. Someone who has exceptionally dense bones to begin with will probably never lose enough calcium to reach the point where osteoporosis occurs, whereas a person who has low bone density could easily develop osteoporosis despite losing only a relatively small amount of calcium.

Type I osteoporosis (postmenopausal osteoporosis) generally develops in women after menopause when the amount of estrogen in the body greatly decreases. This process leads to an increase in the resoprtion of bone (the bones loses substance). Type I osteoporosis occurs in 5% to 20% of women, most often between the ages of 50 and 75 because of the sudden postmenopausal decrease in estrogen levels, which results in a rapid depletion of calcium from the skeleton. It is associated with fractures that occur when the vertebrae compress together causing a collapse of the spine, and with fractures of the hip, wrist, or forearm caused by falls or minor accidents. Type 1 account for the significantly greater risk for osteoporosis in women than in men.

Type II osteoporosis (senile osteoporosis) typically happens after the age of 70 and affects women twice as frequently as men. Type II osteoporosis results when the process of resorption and formation of bone are no longer coordinated, and bone breakdown overcomes bone building. (This occurs with age in everyone to some degree.) Type II affects trabecular and cortical bone, often resulting in fractures of the femoral neck, vertebrae, proximal humerus, proximal tibia, and pelvis. It may result from age-related reduction in vitamin D synthesis or resistance to vitamin D activity (possibly mediated by decreased or unresponsive vitamin D receptors in some patients). In older women, types I and II often occur together.

1.3.4 Secondary Osteoporosis

Secondary osteoporosis is caused by other conditions, such as hormonal imbalances, certain diseases, or medications (such as corticosteroids). Details on the many other causes of secondary disease are included throughout this report. Secondary osteoporosis accounts for < 5% of osteoporosis cases. Causes include endocrine disease (eg, glucocorticoid excess, hyperparathyroidism, hyperthyroidism, hypogonadism,

hyperprolactinemia, diabetes mellitus), drugs (eg, glucocorticosteroids, ethanol, dilantin, tobacco, barbiturates, heparin), and miscellaneous conditions (eg, immobilization, chronic renal failure, liver disease, malabsorption syndromes, chronic obstructive lung disease, Rheumatoid Arthritis, sarcoidosis, malignancy, **prolonged weightlessness as found in space flight**).

1.4 Bone Tissue:

Bone tissue has ability to adapt its mass and morphology to functional demands, its ability to repair itself without leaving a scar, and its capacity to rapidly mobilize mineral stores on metabolic demand; it is in fact the ultimate "smart" material [26]. Mature compact bone contains about 2% of its volume of cells, the remainder being extracellular matrix. Of this matrix 70% is occupied by mineral, about 20% is organic and the remainder is water. Of the organic material 90% is collagen about 1% is proteoglycan and the rest is a series of matrix proteins.

Bone is composed of two forms of bone tissues: cortical (compact) bone and cancellous (trabecular or spongy) bone. Although cortical and cancellous bones possess the same matrix composition and structure, the matrix of cortical bone is 80 – 90% calcified while that of cancellous bone is only 15 - 25% [Garner,S.C. et al,1996]. Thus, cortical bone is denser or less porous than cancellous bone. Cortical bone provides a mechanical and protective role for bone, forming the shafts of long bones and covering nearly all other bones in the body. However, cancellous bone fills the ends of long bones and comprises most of the structure of vertebrae bones . Cortical and cancellous bone may consist of two other forms of bone tissues: woven (fiber or primary) bone or lamellar (secondary) bone. Woven bone is a rapidly formed, poorly organized tissue consisting of collagen fibers and mineral crystals randomly arranged. In contrast, lamellar bone is a slowly formed, well-organized tissue consisting of parallel layers of matrix crystals and collagen fibers . Woven bone is present when bone is first formed, such as in the embryonic skeleton, or during fracture repair. Lamellar bone, however, is present after bone is remodeled during growth or during normal bone turnover; thus lamellar bone is present in mature bone.

1.5 Bone Cells

Mature compact bone contains about 2% of its volume of cells. Bone is composed of several cell types that execute the functions required to form and maintain bone structure. These cells also perform the homeostatic roles of bone. *Osteoblasts, osteocytes, bone lining cells*, and *osteoclasts* are the four cell types of which bone is composed. These cells can be divided into two functional categories: those that form bone and those that resorb bone

1.6.1 Osteoblasts: The principal cell that contributes to bone formation is the osteoblast, which produces and secretes the structural components of the bone matrix (notably **type I collagen**) and releases minerals, growth factors and enzymes into it. Osteoblasts are capable of forming osteoid (unmineralized bone matrix) at a rate of approximately one micrometer per day. These bone forming cells express the **membrane protein alkaline phosphatase**, an enzyme that is believed to be a regulator of mineralization. Osteoblasts also assist in the homeostasis of calcium by possessing receptors for the two primary calcium and bone regulating hormones, parathyroid hormone and 1,25-dihydroxyvitamin D. Osteoblast also produced most abundant non-collagenous protein of bone i.e Osteocalcin or bone Gla protein.

The osteoblast is derived from a MESENCHYMAL stem cell that differentiates into a pre-osteoblastic stromal cell and then into an osteoblast [S.Mann et al,2001] Active osteoblasts may follow one of three courses once bone has been formed: 1) enclose themselves in bone, forming osteocytes, 2) remain on the bone surface, forming bone-lining cells, or 3) disappear from the site of bone formation by undergoing apoptosis .

1.6.2 Osteocytes The osteocyte is the most abundant cell type in mature bone tissue and approximately 90% of the bone cell are osteocytes.They are lying within small cavities known as lacunae that are completely immersed in bone. These former osteoblasts comprise more than 90% of the bone cells in mature bone [Buckwalter,J.A. et al1996].

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Osteocytes remain associated with each other and cells on the bone surface by communicating via small tubular channels known as canaliculi. These tunnels (canaliculi) provide a means for the diffusion of nutrients from extracellular fluid to these cells [Bassett,C.A.L. *et al*,1993]. **Bone-lining cells** are osteoblasts that are no longer actively materializing bone, these cells lie directly on the bone matrix with cytoplasmic extensions that infiltrate the bone matrix and contact the cytoplasmic extensions of osteocytes. These bone-lining cells are believed to be central in the maintenance of blood calcium levels, although the manner in which this occurs remains speculative . Bone-lining cells appear to retain their receptors for parathyroid hormone, contracting and secreting enzymes that remove the thin layer of osteoid covering the mineralized matrix when exposed to this hormone. These actions appear to allow for osteoclasts to attach to the bone surface and begin bone resorption. Thus, bone-lining cells may have a role in attracting and stimulating osteoclasts to resorb bone

1.6.3 Osteoclasts

Osteoclasts are multinucleated, non-dividing,motile giant cells with the specific capacity to resorb mineralized bone tissue. (Bassett,C.A.L.,*et al* 1979) This is unique feature for the osteoclasts. These cells are formed from the fusion of monocytes derived from the hemopoietic portion of bone marrow. . Several enzymes that are released by osteoclasts might participate in matrix degradation; however, cathepsin K, a lysosomal cysteine protease, seems to be selectively expressed in osteoclasts and can degrade type I collagen efficiently(Teiti,A *et al* 1992).

1.7 Bone Matrix

Bone matrix consists of an organic phase and an inorganic phase. The **organic phase** contributes to approximately 20% of the wet weight of bone, with the **inorganic phase** contributing 65% and **water** contributing approximately 10% [Buckwalter, J.A. *et al* 1996].

The **organic phase** of bone, which primarily consists of collagen, provides bone its form and its ability to resist tension. The inorganic phase, however, is the component of bone that gives it rigidity and its characteristic ability to resist compression . The organic matrix of bone is similar to the fibrous matrix present in tendons, ligaments, and joint capsules. Type I collagen is the predominant collagen present in bone, while minor amounts of types V and XII also exist. Collagen makes up approximately 90% of the organic matrix, with noncollagenous proteins contributing to approximately 10%. The noncollagenous proteins present in bone are either synthesized by osteoblasts or serum derived; these proteins may have a role in influencing the organization of the matrix, the calcification of bone, and/or the activities of bone cells . Thrombospondin, fibronectin, bone sialoprotein, osteopontin, proteoglycan I and II, osteonectin, osteocalcin, and matrix gla-protein are some of the noncollagenous proteins included in bone . Growth-related proteins have also been identified in bone, including the transforming growth factor-β family, insulin-like growth factor-1 and 2, bone morphogenic proteins, platelet-derived growth factors, interleukin-1 and 6, and colony-stimulating factors Although the specific function of these proteins remains uncertain, the fact that they are incorporated into bone suggests that they have an important role in bone.

Osteocalcin is the most abundant noncollagenous protein in bone1, and its concentration in serum is closely linked to bone metabolism and serves as a biological marker for the clinical assessment of bone disease. Although its precise mechanism of action is unclear, osteocalcin influences bone mineralization in part through its ability to bind with high affinity to the mineral component of bone, hydroxyapatite[S.Mann, 2001]. In addition to binding to hydroxyapatite, osteocalcin functions in cell signalling and the recruitment of osteoclasts and osteoblasts, which have active roles in bone resorption and deposition, respectively. The X-ray crystal structure of porcine osteocalcin at 2.0A ° resolution, which reveals a negatively charged protein surface that coordinates five calcium ions in a spatial orientation that is complementary to calcium ions in a hydroxyapatite crystal lattice.

The **inorganic matrix** of bone, or the mineral phase, serves as an ion reservoir for the body, containing approximately 99% of the body calcium, 85% of the phosphorus, and 40

to 60% of the total body magnesium and sodium . It is through this function as an ion reservoir that bone is able to maintain the extracellular fluid concentration in a range necessary for critical physiological functions. The inorganic phase consists primarily of hydroxyapatite crystals, $C_{10}(PO_4)_6(OH)_2$, comprising 60 − 65% of bone weight . Minor amounts of sodium, potassium, magnesium, citrate, carbonate, fluoride, and other ions have also been shown to substitute or be absorbed onto the crystal surface. A substitution of ions present on hydroxyapatite with other ions is governed by the composition of the extracellular fluid, and in turn, affects the solubility of the mineral phase.

1.7 Bone water occurs at various locations and in different binding states. It is associated with the mineral phase, bound to the organic phase (collagen and cement substance) and a large fraction occurs in more or less free form (Bulk water). The "Bulk water" fills the pores of the calcified matrix making up the Haversian and lacuno-canalicular system. It is this fraction of water that has been shown to confer the unique viscoelastic properties to bone, largely lost after drying. In its natural fully hydrated state, the stress-induced deformation upon application of a load is damped by the resistive forces experienced by the fluid in the lacuno-canalicular system [Hu, P. Y. et al 1992]. The most tightly bound water is the one occupying the calcium ion coordination sites in the apatite-like crystals (about 35 mg of water/g mineral) [Teti,A. et al 1989]. This water cannot be displaced by simple drying at 100° C. A significant, less tightly bound fraction is water associated with collagen fibrils.

1.8 Bone Formation

Although all bones are created by the placement of hydroxyapatite crystals within a collagen matrix, the process by which the collagen matrix is arranged differs depending on the type of bone formation utilized. Two types of bone formation exist: intramembranous and endochondral bone formation (Garner, Anderson, Ambrose, 1996).

Intramembranous bone formation is utilized to form the flat bones of the skull, jaw, and

ribs. During this process, mesenchymal cells differentiate into preosteoblasts and then into osteoblasts. The bone tissue formulated by these cells is woven, causing calcification in this tissue to be in irregular patches due to the irregular arrangement of the collagen fibers. As these bones undergo tissue remodeling, the woven bone will be replaced by lamellar bone (Garner, Anderson, Ambrose, 1996).

Endochondral bone formation is utilized to form long bones; this process involves the calcification of cartilaginous structures. The formation of cartilage is performed by chondroblasts, cells that are derived from mesenchymal cells, which differentiate to form prechondroblasts, and then form chondroblasts. The chondrocytes secrete a collagen matrix, which eventually encloses them into lacunae. Although they are enclosed within these small cavities, the flexibility of the collagen fibers surrounding them allows for the chondrocytes to continue to proliferate and differentiate, expanding the lacunar spaces. The invasion of blood vessels into the cartilage initiates the mineralization process of the organic matrix. The mineralization decreases the ability of nutrients to diffuse through the organic matrix, causing the chondrocytes to begin to die. Only a thin layer of chondrocytes, at each end of the bone, survives the initial mineralization. The remainder of these active chondrocytes allows for bone to continue elongating at this site, a site known as the epiphyseal growth plate (Garner, Anderson, Ambrose, 1996).

1.9 Mineralization

The formation of crystals in an aqueous solution, a process necessary for bone mineralization to occur, involves three primary physico-chemical steps: supersaturation,

nucleation, and crystal growth or maturation (Perry, Fraser, Hughes, 1991). The supersaturation of an ion occurs when the activity product of the ion exceeds the solubility product, favoring the precipitation of that ion. The nucleation of that

precipitated mineral involves the deposition of the mineral onto an active interface, such as that provided by an organic substrate. The growth of the mineralization process involving matrix vesicles is theorized to occur in two distinct phases: initiation of first crystals and regulation of crystal proliferation (Anderson, 1985).

In the first phase, calcium is believed to accumulate in the matrix vesicles prior to phosphate due to their attraction to the acid phospholipids concentrated within the vesicle. Phosphate levels then appear to increase in the matrix vesicle via the action of phosphatases, allowing for the deposition of solid phase calcium phosphate minerals to occur.

In phase two, crystal proliferation is believed to be initiated by the exposure of the preformed apatite crystals to extracellular fluid. Crystal proliferation continues to occur until it eventually infiltrates the surrounding matrix.

Another theory states that collagen fibrils are the nucleation catalysts of mineralization, containing specific nucleation sites that allow for mineralization to occur (Glimcher, 1985). Phosphoproteins residing within the collagen fibrils are also believed to have an important role in the mineralization process, by possibly binding calcium and phosphate ions, facilitating their conversion to a solid phase. Inhibitors of crystal proliferation include: inadequate levels of calcium and phosphate; certain organic phosphate compounds, which prevent hydroxyapatite growth, like pyrophosphate and ATP; specific noncollagenous proteins; and γ-carboxyglutamic acid-containing proteins, proteins which have been found to impede hydroxyapatite deposition. Factors seen as promoting crystal proliferation include: elevated calcium and phosphate levels, collagen, and osteonectin.

(Garner, Anderson, Ambrose, 1996).

1.10 .1 Collagen

This section briefly introduces the collagen family and identifies the structure of collagen, the types of collagen, and their classifications. A focus on type I collagen is also

made in this section because of its prominence in bone. The biosynthesis, intracellular alterations, and extracellular processing of type I collagen will be described.

Collagen Family

Collagen accounts for approximately 30% of the total protein present in mammals, making it the most abundant protein in mammalian tissues (Yamauchi, 1996). Its presence in the extracellular space in nearly all types of connective tissue in the body makes it a key structural protein. Collagen possesses a triple-helical structure, with each polypeptide chain (α chain) consisting of a repetitive sequence of amino acids (glycine-proline-hydroxyproline) (10Nimni, 1988).

The presence of glycine, the smallest amino acid, in the third position of the repeating sequence is important for the helical structure of collagen because it occupies the center of the helix. Thirty unique alpha chains have been identified. Nineteen unique collagen types have also been identified; each type is distributed in a characteristic tissue with a unique biological function.

Three different classifications exist for the types of collagen: fibril-forming, fibrilassociated, and nonfibrillar collagens (11Yamauchi, 1996). Types I, II, III, V, and XI are fibrilforming collagens. These collagen types, as their name implies, form fibrils in various tissues in which they are found. They are rod-shaped molecules, packed in parallel lines, staggered longitudinally in respect to one another. This packing arrangement creates areas that have high and low densities of collagen, forming areas known as hole zones (gaps), and overlapping zones (Figure 2.). The fibril-associated collagens, however, do not form fibrils; these collagen types are believed to function as regulators of the fibril diameter and/or as bridges between fibrils. Types IX, XII, XIV, and possibly XIX are fibril-associated collagens. Several nonfibrillar collagen types have been identified, in addition to the fibril-forming and fibril-associated collagens. Types IV, VI, VII, and X are nonfibrillar collagens.

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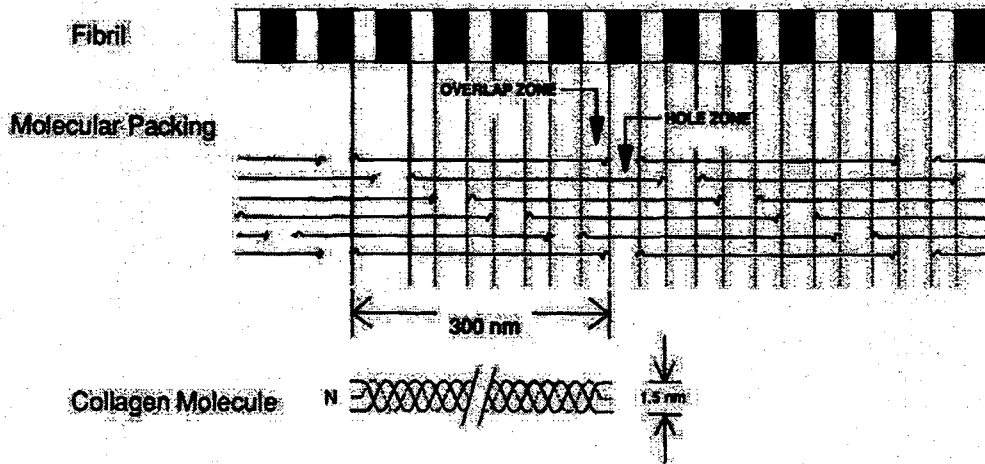
Figure 2. A two dimensional diagram of a type 1 collagen fibril. Yamauchi, M. (1996) Collagen: the major matrix molecule in mineralized tissues. In: *Calcium and Phosphorus in Health and Disease.* (Anderson, J.J.B., Garner, S.C., eds.), pp. 129, CRC Press Inc, Boca Raton,Florida.

Synthesis of Type I Collagen

Type I collagen is composed of three alpha chains, two α-1 chains and one α-2 chain

(Nimni, 1988). The synthesis of type 1 collagen is a complex, multiple-step process that involves both intracellular and extracellular modifications (Figure .3). In order for the development of extracellular collagen fibers, a cell must first synthesize procollagen, a precursor molecule to type 1 collagen. The procollagen molecule consists of three different domains: the NH2-terminal, the triple helical, and the COOH-terminal domains. The triple helical domain represents approximately 95% of the procollagen molecule (Yamauchi, 1996). Once procollagen is synthesized and released from the cell it is enzymatically cleaved of both of its nonhelical ends, to produce a collagen molecule that spontaneously constructs into fibers in the extracellular fluid.

ROUGH ENDOPLASMIC
RETICULUM
Procollagen chain synthesis &
modifications
- Hydroxylation of proline and
  lysine
- Glycosylation of OH-lysine

- Chain association
- Disulfide bond formation
- Nucleation & Propagation

Triple helix formation

(Procollagen)

Secretion

▲ Enzymatic cleavages
(N- and C- proteinases)

N   C

(Collagen)

Fibril Formation,
lysyl/OH-lysyl
oxidation &
cross-linking

O-Gal-Gln
α1 chain
α2 chain

INTRACELLULAR

EXTRACELLULAR

Figure .3. A schematic representation of the formation of collagen. Yamauchi, M. (1996)
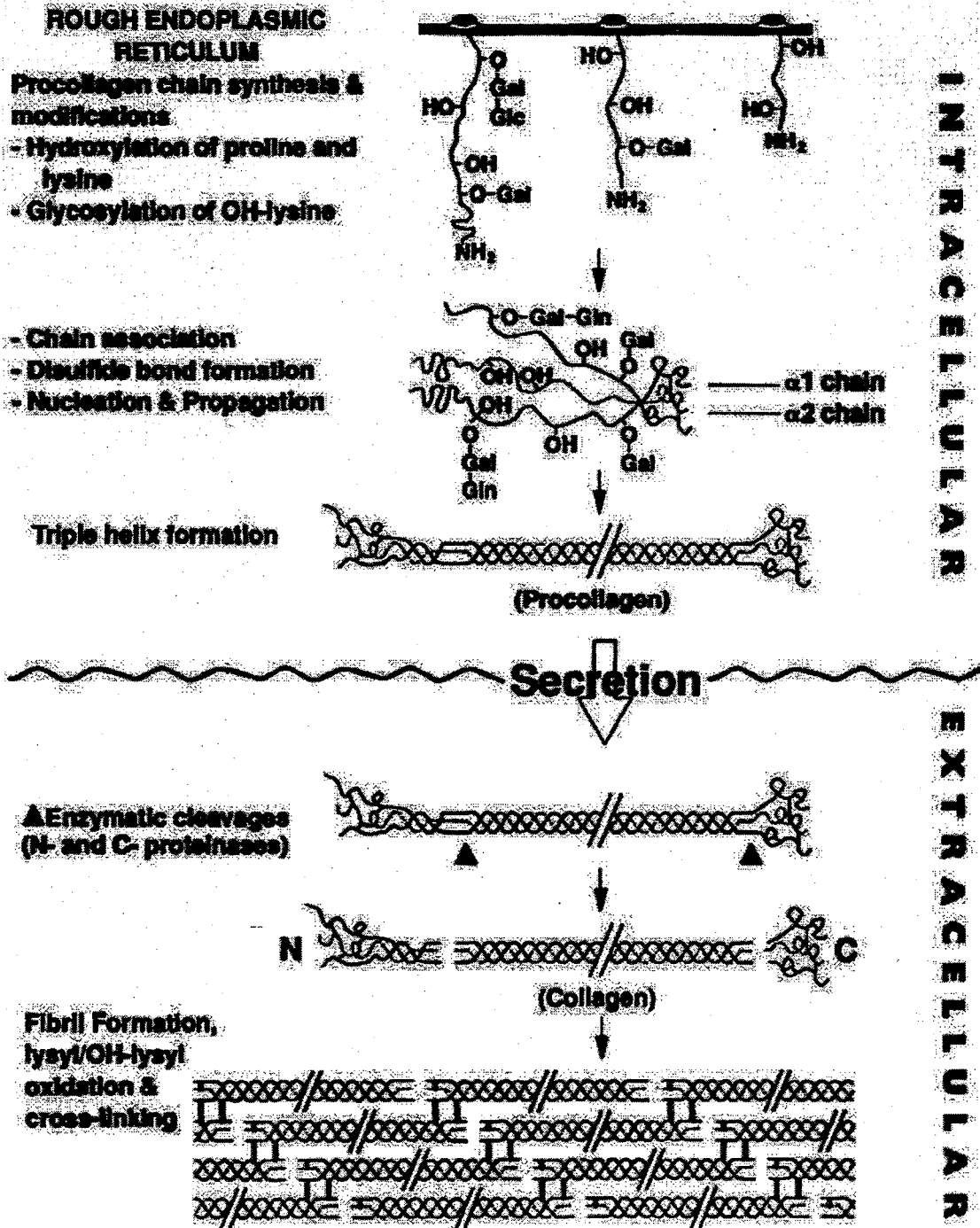Collagen: the major matrix molecule in mineralized tissues. In: *Calcium and Phosphorus*

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in Health and Disease. (Anderson, J.J.B., Garner, S.C., eds.), pp. 132, CRC Press Inc, Boca Raton,Florida.

The synthesis of intracellular procollagen is initiated by the transcription of the collagen gene (Nimni, 1988). Specific messenger RNAs are formed for each alpha chain of the collagen molecule and translocated from the nucleus into the cytoplasm. Once in the cytoplasm, the messenger RNA is translated in the rough endoplasmic reticulum on membrane-bound ribosomes. After the translation of the single pro-alpha chains, the chains are transferred into the lumen of the rough endoplasmic reticulum where essential cotranslational events occur. The hydroxylation of specific proline residues by 3-proline hydroxylase and 4-proline hydroxylase and of lysine by lysyl hydroxylase occurs during this time, forming hydroxyproline and hydroxylysine respectively. This modification occurs prior to the formation of the triple helix, since the enzymes that mediate this reaction are inactive against a triple helical structure (Yamauchi, 1996). These three enzymes also require ferrous iron, ascorbate, and α-ketoglutarate for activity. As the lysyl residues are hydroxylated, sugar residues are also added. Two enzymes, galactosyltransferase and glucosyltransferase, catalyze this glycosylation reaction, with the first enzyme adding galactose to the hydroxylysine residue and the second adding glucose to the galactosylhydroxylysine residue that was formed. These two enzymes, similar to the hydroxylation enzymes, require the pro-alpha chains to be in the nonhelical conformation. A bivalent cation, preferably manganese, is also required to be present during these reactions. Once these modifications and additions are complete, it is essential for pro α chains to properly align for the triple helix to form. The COOH-terminal propeptides initiate the intertwinement of the three pro α chains through the formation of disulfide bonds between the individual pro α chains. This association allows for the spontaneous formation of the triple helical structure, an intertwinement that propagates from the COOH-terminal to the NH2-terminal. Once the procollagen molecule is formed, it travels from the lumen of the rough endoplasmic retiuclum through the transitional endoplasm to the Golgi apparatus. In the Golgi, procollagen is packaged

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in saccules, transformed into secretory granules, and extruded into the extracellular space via a cellular process known as exocytosis.

Once released from the cell, extracellular processing is required to transform procollagen into the active fibril-forming type I collagen. The C- and N-terminal propeptide extensions of the procollagen molecule are cleaved by the enzymes procollagen C-proteinase and procollagen N proteinase, respectively (Nimni, 1988). Once these two procollagen extensions are cleaved, the type I collagen molecules spontaneously aggregate to form a fibril (Yamauchi, 1996).

1.10.2 Bone Collagen Cross-links

The orientation of the collagen molecules in the fibril consists of a staggered longitudinal pattern of molecules with the multiples having tissue-specific lateral orientations with respect to one another. The cross-linking of bone collagen is initiated by the conversion of a specific peptidyl hydroxylysine to its aldehyde form, hydroxyallysine. This conversion occurs almost immediately after the collagen molecule is synthesized and is catalyzed by lysyl oxidase, a copper dependent amine oxidase enzyme that requires oxygen and a carbonyl cofactor for activity. The orientation of the hydroxyallysine is near the "hole zone" and reacts with the adjacent hydroxylysine to form a bifunctional cross-link prior to mineralization. These links are able to tautomerize between the iminium and keto forms. This bifunctional cross-link is stereospecific in nature, connecting adjacent molecules laterally, in a sheet-like manner. The cross-links also laterally join neighboring hole regions, forming hole zones with plate-like shapes. It is this nature of cross-links that leads researchers to speculate the dynamic role collagen has in mineralization, regulating the size, shape, and site of mineral crystals within the fibrils (Yamauchi, 1996).

CHAPTER-2

LITERATURE REVIEW

It is known that weightlessness during space flight causes osteoporosis. The loss of bone mineral density (BMD) in the spine,femoral neck and trochanter and pelvis is 1.0 - 1.6 % in the spine per month(Jun Iwamoto *et al*) Osteoporosis after space flight may lead to and increased risk of fracture in later life .

Microgravity changes the metablic environment of bone leading to site-specific alterations in bone remodeling; bone formation is decreased and bone resorption is increased resulting insignificant bone loss with an attendant increase in fracture risk. (J.R.Shapiro *et al* 2006) The effect of calcium , Vitamin d, and vitamin k on calcium balance and bone metabolism during space flight in astronauts have been reported, and the efficacy of bisphosphonates,parathyroid hormone (PTH) ,testosterone, and vitamin K2 for bone metabolism and bone mass has also been studied in the tailed-suspended rat as a model of astronauts. The effect of exercise on bone metabolism and bone mass has rarely been reported, although its efficacy is expected.Immobilisation (e.g. bed rest or restricted movement of limbs) can also caouse rapid loss muscle or bone mass (Rich KM *et al* 1991) In addition , these condition s lead to negate calcim balance and loss of bone mineral density (BMD) . Siilar reductions in musculoskeletal mass have been reported associated with immobilization of extremities seen in external bandaging, casting or neural re-sectioning. Both mineralization and collagen metabolism seem to be impaired in animals during the first few days of space flight. Reductions in muscle forces led to decrease in bone formation and BMD in the os calcis and increase in urinary loss of calcium, but no loss of BMD in the radius

Smith et al (Jun Iwamoto *et al*) analyzed numerous reports on changes in bone metabolism during space flight without any interventions such as medication and exercise . Weight-lessness during space flight without any interventions such as medication and exercise. Weight-lessness during space flight increases urinary calcium excretion, decreases intestinal calcium absorption,and increases serum calcium level , with decreased levels of serm PTH and calcitriol. Bone resorption is increased as indicated by increased urinary pyridinoline,deoxypyridinoline and cross-linked N-terminal telopeptide

of type 1 collagen and urinary and serum cross-linked c-terminal telopeptide of type 1 collagen levels ,whereas bone formation is decreased serum bone-specific alkaline phosphatase and c-terminal peptide of type 1 collagen and urinary and serum cross-linked c-terminal telopeptide of type 1 collagen levels, wherea bone formation I ecreased as indicated by ecreased serum bone-specific alkaline phosphatase and C-terminalpeptide of type 1 procollagen levels, followed by increased serum undercarboxylated osteocalcin level.

Micrograity induces changes in growth, cellular structure, and cell-to-cell interactions.(Poonam Sarkar *et al,* 2006) These changes are therefore considered to be a challenge to life in space. Exposure to altered microgravity during space flight has long-lasting effect on postural stability and cardiovascular function .In the brain the activity of neural functions has been a concern as it relates to the fluidic nature of the gray and white matter because of its enclosure within skull. The sensitivity of the mammalian central nervous system to gravitational influences involves both direct and indirect factors. It has been reported that changes in the gravitational environment might represent a useful tool to investigate the neurological and behavioral responses to stressors and may provide insights into the mechanisms underlying development and plasticity of the nervous system in rain , heart an lunch tissue.(Poonam Sarkar *et al* 2006)

It has also been reported that mechanical unloading may also account for the differential loss in musculoskeletal masses of small animals flown in space flights(Reich KM 1991) Over the past few years, several countermeasures have been examained to prevent the loss of musculoskeletal mass during exposure to microgravity.

2.1 Calcium Balance in Humans in Microgravity Environment

Travel within the microgravity environment of space causes many physiological responses that can be detrimental to astronauts' health upon their return to earth. One aspect that is of great concern is changes in bone deposition and resorption rates that can lead to losses in bone mineral density (BMD). Early human spaceflight studies have demonstrated increased calcium excretion through the urine (Rambaut P.C. *et al* 1979)

and feces . During the Skylab II mission, crewmen consumed a controlled diet and collected urine and fecal excrement to determine changes in calcium balance during spaceflight. Urinary calcium increased steadily to approximately double the pre-flight values by day 28 while fecal calcium did not change significantly. The result was a mean calcium loss of 50 mg/day during the last 16 days of flight (Whedon G.D *et al* 1975). To further demonstrate the changes in calcium metabolism, Rambaut et al. (Rambaut P.C. *et al 1979*) combined data from several orbital spaceflight missions occurring over 28, 59, and 84 days in which diet was controlled and excrement was monitored. During the first 28 days calcium excretion in the urine increased to approximately 100% of pre-flight levels then leveled off for the remainder of the flight. Fecal calcium showed an initial decline at 14 days then increased steadily throughout the 84 day period to approximately 300 mg/day above pre-flight values . Calcium balance showed a steady decline throughout the 84 day period to a maximum deficit of approximately 300 mg/day .

It is hypothesized that a negative calcium balance is related to the decreases in bone mineral density observed as a result of microgravity. Bone mineral density has been shown to decrease at rates between approximately 1%-2% a month in specific bones (Holick M F ,1998). The decreases in bone mineral density have been observed primarily in the weight bearing bones of the calcaneus, lumbar spine, and tibia

2.2 Study of Simulated Microgravity on Rats

Conducting research during space flight can pose many challenges. Space missions are limited in number and duration, require many man hours, and are expensive. The development of a ground-based model to simulate microgravity is important in the study of the effects of the microgravity environment incurred in humans during space travel. The benefits of ground based models are: (a) scheduling times for experiments are not limited to the number of space missions, (b) not as many precautions are needed during experimental manipulations on Earth, (c) ground-based experiments are more economical, (d) experimental duration is not limited to mission duration, (e) measurements can be made and tissue samples can be obtained at several points during the experiment, and (f) experiments can be extended or repeated on a regular basis

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(Morey-Holton E.R. *et al*,2002).

The physiological responses to space travel include fluid shifts, repositioning of certain organs, musculoskeletal unloading, and lack of stimulus to the vestibular system (Morey-Holton E.R. *et al*,2002). Human models have successfully simulated these effects by using bed rest and bed rest with a head-down tilt. Human ground-based models have their own limitations, therefore, animal models have been developed which successfully simulate the effects of space flight. A common animal model is the hindlimb suspended (HLS) rat model which has been approved by National Air and Space Administration (NASA). The HLS model requires that the rat's hindlimbs be suspended so the body axis creates approximately a 30° angle with the floor. This allows for normal weight bearing on the forelimbs . The HLS model presents minimal stress to the animal based upon hormonal levels and body weight measured(Morey-Holton E.R. *et al*,2002). Suspension is achieved by using a tail traction harness which allows the rat freedom to move, eat, and groom.

2.3 Bone Mineral Changes in Rats Exposed to Simulated Microgravity

Bone mineral loss during HLS in the rat model has been shown to occur in the femur, lumbar vertebrae, and tibia. Increased bone mineral deposition has been observed in the humerus, skull, and mandible. Arnaud et al. (Bassett, C.A.L. *et al* 1979) suspended 6-month-old rats for 4 weeks and measured bone mineral content *in situ* of the whole body and the femur and skull separately using dual x-ray absorptometry (DEXA) technique. Bone mineral content, when corrected for body weight, was 14% and 7% lower in whole body and femur, respectively. There was no significant change in the skull bone mineral content (Bassett, C.A.L. *et al* 1979). Roer and Dillaman (Roer R.D. *et al,* 1990) calculated the differences in wet, dry, and ash weights of the forelimbs, hindlimbs, and the skull during 1-, 2-, and 3-week HLS studies on 28-day-old rats. For the 3-week experiment, when corrected for body weight, the dry and ash weights of the femur and tibia were significantly lower ($p < 0.05$ for dry tibia; $p < 0.001$ for dry and ash femur, and ash tibia) in the HLS group. The dry weights of the forelimbs were greater ($p < 0.01$) in the HLS group, however there was no difference in ash weight, while the dry and ash

weights of the skull and mandible were greater ($p < 0.001$) in the HLS group (Dehority W. *et al* 1999).

Dehority et al. (1999) measured the bone weight using regression analysis in 6-month-old HLS and control rats in three separate experiments of 1-, 3-, and 5 week durations. There were significant decreases ($p < 0.05$) in the regression slopes for the fat-free weights of the femur and lumbar vertebrae but not for the humerus. Globus et al. (1984) measured changes in calcium in the humerus, mandible, tibia, and lumbar vertebrae after 15 days of HLS. The tibia and lumbar vertebrae of the HLS rats contained $86.2 \pm 2.5\%$ and $75.5 \pm 3.5\%$ (mean $\pm$ SD) calcium of the control values respectively. There were no differences in calcium content in the mandible and humerus between the HLS and control groups

Bloomfield et al. (2002) used peripheral computed tomography (pQTC) to compare the differences in BMD of the mid-diaphysis and proximal metaphysis of the tibia in six-month old rats after 7-, 14-, 21-, and 28-days of HLS. (Peripheral quantitative tomography separates cortical and cancellous bone mineral compartments and provides a real measure volumetric density (mg/cm^3).) When compared to 0-day control both the 28-day HLS and 28-control exhibited a similar increase in cortical BMD of the proximal tibia, $+5.7\%$ and $+4.0\%$ respectively. Cancellous BMD decreased by 21% after 28-days HLS in proximal tibia when compared to 0-day control. Greater BMD was observed at the tibial mid-diaphysis at days 14 – 28 of HLS. No significant difference was observed when BMD values were compared to 28-day control.

2.4 Systemic and Local Factors Contributing to Bone Loss in microgravity

The regional decreases in hindlimb mass and bone mineral density and the increases or stabilization of the skull and forelimb mass and bone mineral density may be attributed to an alteration in perfusion of bone as a result of the cephalad fluid shift during HLS rather than systemic factors that regulate bone mineral homeostasis(Dehority W., B.P. Halloran *et al* 1999). In a study by Dehority et al. (1999), changes in PTH were not observed after 5 weeks of HLS, however there were regional changes in MAR and BFR, therefore the data did not indicate that systemic factors contribute to bone loss (Dehority W., B.P. Halloran *et al* 1999). It is believed that if systemic factors influenced bone loss during

simulated microgravity, then changes in BMD would appear in all areas of the body.

2.5 Biochemical Markers of Bone Resorption

Biochemical markers can be used as an indicator of the rate of bone turnover. During bone formation type I collagen is laid down then bonded together by pyridinium and pyrrolic cross-links for structural integrity (Knott L., et al ,1998). During resorption the collagen is broken down by osteoclasts then pyridinoline (PYD) and deoxypyridinoline (DPD) are released. Pyridinoline and DPD are neither metabolized nor absorbed from the diet (Eastell R.., et al 1997) and can be measured in the blood or urine. Low levels of resorption markers, such as DPD, reflect low rate of bone resorption and high levels of DPD reflect a high rate of bone resorption activity. A high rate of bone loss can lead to a decrease in bone mineral density and osteoporosis. Moderate correlations have been observed between high concentrations of bone resorption markers and bone mineral loss . Caillot-Agusseau et al. (1998 and 2000) observed a 50 % and 54 % increase in DPD in cosmonauts during 21 and 180 days of spaceflight respectively. Kurokouchi et al. (1995) measured changes in tartrate-resistant acid phosphatase (TRAP), a bone resorption marker, in five-week old rates during HLS. The results indicated a significant increase in TRAP at days one and three, but returned to pre-HLS level at day five and remained there throughout the duration of the study.

2.6 Biochemical Markers of Bone Deposition

Osteocalcin (OC) is a non-collagen protein excreted by exclusively osteoblasts into the bone matrix during bone deposition Some osteocalcin escapes the bone matrix entering the bloodstream and is a good indicator of osteoblast activity (IMMUTOPICS). The literature suggests that bone formation is reduced during space travel and simulated microgravity. Caillot-Agusseau et al. (Caillot-AugusseauA., et al 1998) measured changes in OC during 21 and 180 day space missions in cosmonauts. Osteocalcin levels were decreased by 18 % and 27 % during 21 and 180 days respectively when compared to pre-flight levels. Kurokouchi et al. (1995) observed a decrease in OC levels from day three to day 14 in 5-week old HLS rats. Patterson-Buckendahl et al. (1989) measured weekly changes in OC levels for four weeks during HLS in 6.5 week-old rats.

Osteocalcin for the control rats gradually decreased throughout the four week period. The HLS rats demonstrated a significantly greater decrease in OC than the control for week one and leveled out at week two but remained significantly lower than the control value. There was no significant difference in OC levels for weeks three and four and the rate of decrease in OC decreased in parallel between the two groups for the remaining two weeks of the experiment.

Biochemical markers for bone resorption or deposition should not be used to predict the rate of bone mineral loss or bone mineral deposition however they may be used to indicate whether bone loss is a result of excess resorption or decreased deposition rates.

2.7 Animal model study

Tail-suspended young growing rats have been well utilized to test the bone metabolism and bone mass loss during weightlessness. Mean periostial apposition rates (MAR) in adult rats (6-months-old) after 2 and 4 weeks of HLS were reduced by 65% and 85% respectively (Dehority W et al ,1999). In the same study mean periostial bone formation rate (BFR) of the tibiofibular junction (TFJ) and the tibia mid-shaft were decreased by approximately 80% ($p < 0.001$), and there were no significant changes in BFR in the humerus (Dehority W et al ,1999). In a separate study periostial MAR and BFR were 61% and 90% lower at 21 days HLS at 21 days in the tibial mid-diaphysis; no significant differences in MAR and BFR were observed in the mid-tibia and humeral diaphysis (Bloomfield S.A. et al 2002). In cancellous bone, a decrease of 33% and 69% ($p < 0.05$) in MAR and BFR respectively was observed after 2 weeks of HLS in adult rats (Reich KM et al). The percent surface area occupied by cancellous bone occupied by osteoblasts decreased by 66% ($p < 0.05$), there were no significant changes in osteoclast numbers or cancellous bone volume . These results suggest that a decrease in BFR or osteoblast activity may account for the net bone loss seen during simulated microgravity

CHAPTER-3

AIMS AND OBJECTIVES

The human skeleton has evolved in the environment of Earth's gravity . This gravitational force play an important rule in the development of our skelton system. Therefore, the removal of gravity during long-duration space flight induces several physical responses such as a fluid shifts from the lower extremities to upper body, repositioning of certain organs, musculoskeletal unloading, and a lack of stimulus to the vestibular system . These adjustments can lead to physiological adaptations that are detrimental to health such as decreases in mineral density in weight bearing bones and orthostatic intolerance. However, several parameter of bone formation and resoption has been examined using hind-limb suspension model in rat as simulated microgravity for different time periods, but there are lot of controversy in the result within the research groups. Also, there is very little study to see the consequences of long term space flight.

With above mentioned background in present work, we made an attempt to expose the animal in simulated microgravity for forty-five (45) days and to evaluate the following parameter to measure the changes in HLS rat as compared to control:

1. Evaluation of Bone Mineralogy: a) BMD

b) BMC

and,

c) Ca and P content

2. Evaluation of the Collagen I formation.

3. Evaluation of Bone Formation marker Alkaline Phosphatase.

4. Scanning electron microscopy was done to evaluate the changes in cortex and cancellous part of bone.

CHAPTER-4

MATERIALS AND METHODS

4.1 Animals: Experimental Methods

Animal model study
Tail –suspended young growing rats

The tail suspension (or hindlimb unloaing [HU]) model in rats is well accepted as the best small animal model for simulating microgravity effects on cardiovascular outcomes as well as musculoskeletal changes in the unweighted hindlimbs The elevated hindlimbs are free to move but can not bear any weight. Importantly, the head-down posture of the tail-suspended rat also induces the headward fluid shifts observed inbiedal humans during spaceflight. .

Female Wistar rats (90 days old and similar mean weight,210-220 gm body wt.) were obtained from animal facility of Jawaharlal Nehru University, New Delhi. They were randomly divided into two groups (Control and HLS,) and housed in an air conditioned room (temperature was maintained at 25°C). They were provided with standard food pellets (Hindustan lever ltd., India) and tap water ad libitum

Fig 4: Hindlimb suspended rat in the cage.

4.2 Mineralogical Analysis

Bone Samples Preparation:

Rats were sacrificed with an intraperitonial overdose of phenobarbital sodium, and tibia and femora of **Control** (Normal rats), and HLS were freed from soft tissues and stored at -20° C for various assays. Volume of all fresh bones were measured by submersion of bone in a water filled container with a scale sensitivity of 0.01 ml. After measuring the volume, bones were lyophilized for 10 hrs and powdered down into fine particles with help of mortar and pestle. Powdered samples were kept at 60° C overnight in vacuum oven for determination of Total Dry Bone Weight (organic + inorganic contents).

4.2.1Mineral and organic content:

Bone samples were kept at 700° C in a muffled furnace (Widsons Scientific Works India) for 10 hrs. The ash weight was indicated as Bone Mineral Content. Difference in dry bone and ash weight measured as organic content and following parameters were calculated:

Bone Mineral Content (BMC) = Ash weight (mg.)

Bone Organic Content (BOC) = Total Dry Bone Wt. - BMC

Bone Mineral Density $BMD = \dfrac{BMC}{BoneVolume}$

{The application of Archimedes principle is standard method for determination of Bone Mineral Density (gm/cm^3).}

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4.2.2 Calcium Analysis:

Calcium was determined in rat bone samples by Atomic Absorption Spectroscopy (AAS). Before analysis, powdered bone samples were digested in acid and then calcium was quantified by flame atomic absorption spectrophotometry (SHIMADZU AA-6800). 50 mg. of bone powder from each sample was digested in 5 ml. of aquaregia and heated in Teflon bomb at 100° C for 10 hrs. Subsequently, the digested samples were diluted with milli Q water and 100 ml stock solution was made. 5 ml of stock solution was added with 95 ml of 0.5% lanthanum chloride and filtered through 0.45 μm pore size filter. The standards of different Ca concentrations (i.e., 1 PPM, 2 PPM, 4 PPM, 5 PPM and 10 PPM) were prepared from commercial standard solution (Ranbaxy, india). The standards and samples were read against the blank solution. The reading of samples, standards and blank were noted. The concentration of calcium in the samples were calculated by equation obtained from the standard curve

Lanthanum Chloride (5% working stock)				
58.64 g of lanthanum oxide (La₂O₃) was added to approximately 50 ml of distilled H₂O. Thereafter 250 ml of concentrated HCl added slowly and diluted to 1 L with distilled H₂O.				
Final ppm desired	Dilution	Amt. 1000 ppm stock	Amt distilled H₂O	Total volume
0.5 %	1/10	100 ml	900 ml	1000 ml
Calcium Standard				
Final ppm Desired	Dilution	Amt. 1000 ppm Stock	Amt. 0.5% LaCl₃	Total volume
1 ppm	1/100	0.5 ml	49.5 ml	50 ml
2 ppm	1/50	1 ml	49 ml	50 ml
4 ppm	1/25	2 ml	48 ml	50 ml
5 ppm	1/20	2.5 ml	47.5 ml	50 ml
10 ppm	1/10	5 ml.	45 ml.	50 ml

4.2.3 Phosphorus Analysis:

Phosphorus analysis of bone samples were performed by Vanado-Molybdo-phosphoric acid colorimetric method in UV-VIS spectrophotometer (Varion AA-20, Varion Analytical Instrument). 50 mg of dry bone powder from each sample was digested in mixture of 1 ml H_2SO_4 and 5 ml of HNO_3 then heated till the solution became 1 ml, for complete removal of HNO_3. Thereafter the solution was neutralized and 100 ml sample stock solution was prepared with double distilled water. 5 ml of sample stock solution was added to 30 ml of double distilled water and 15 ml of Vanadate-molybdate reagent (VMR) and filtered through 0.45 μm pore size filter paper (Millipore). Phosphorus concentrations were evaluated with respect to standard solutions of 0.2 PPM, 0.4 PPM, 0.6 PPM, 0.8 PPM and 1 PPM concentration from phosphate (KH_2PO_4) standard stock solution. The standards and samples were read against the blank solution at 470 nm wavelength. The reading of samples, standards and blank were noted. The concentration of phosphorus in the samples were calculated by equation of standard curve (R^2=0.9976).

Reagents

Vanadate-Molybdate Reagent (VMR):

Solution A: 25 g of ammonium molybdate was dissolved in 400 ml distilled water.

Solution B: 1.25 g of ammonium metavanadate was dissolved in 300 ml distilled water by heating up to boiling temperature and then cooled to room temperature and 330 ml of concentrated HCl was added to it. Solution A poured into Solution B and diluted to 1L.

Standard phosphate solution (1.00 ml = 50 μg PO_4 - P): 219.5 mg anhydrous Potassium dihydrogen phosphate (KH_2PO_4) was dissolved in distilled water and diluted to 1 Liter.

4.3.1 SEM (Scanning Electron Microscopy) Analysis

Femur and tibia of each group were dissected and removed all soft tissues. Transverse sections of 0.5 cm thickness of femur and tibia were made in each bone sample (CONTROL, and HLS). They were fixed in 2% Glutaraldehyde. Before doing any SEM characterization, the bone samples were dried and mounted on circular disc stubs with adhesive. Gold/Carbon coatings were applied at a thickness of about 20 nanometers, which is too thin to interfere with dimensions of surface features. Coating was done with the help of sputter coater. The samples were placed in a small vacuum chamber. After introducing the argon gas in the chamber, electric field was applied to cause removal of electron from the argon atoms and made them positively charged. The Ar ions were then attracted to a negatively charged piece of coated material. The Ar ions acted like sand in a sandblaster, stricken gold or carbon atoms from the surface of the foil. These gold atoms now settled onto the surface of the sample, produced a gold or carbon coat. SEM images were obtained on 'low vacuum SEM' *Leo 435 VP* (Cambridge, England) at National Facilities of Electron Microcopy, AIIMS, New Delhi. The scale present in the SEM images was used to measure the cortical thickness of bone.

4.4.1 Collagen I Estimation in Bone Samples

Bone samples were decalcified with EDTAG (0.5 M EDTA in 0.05 M Tris/HCl) and homogenized in 2 ml 0.05 M Tris/HCl with proteinase inhibitor. Homogenized samples were lyophilized in vacuum drier for 8 hrs. Lyophilized samples were extracted with 2 ml of 0.5 M acetic acid for 48 hrs. The acetic acid soluble collagen was separated by centrifugation at $15000g$ for 45 min. Supernatant of centrifuged material was transferred in a microcentifuge tube and the residue was subjected to subsequent digestion with pepsin (1 mg of pepsin/10 mg of lyophilized bone) in 2 ml of 0.5 M acetic acid (pH 2.0) for again 48 hrs. The pepsin-soluble collagens were separated by centrifugation at $15000g$ for 45 min. The pepsin-insoluble bone matrix was extracted with 0.05 M Tris/HCl, pH 7.5, containing 4.0 M guanidine hydrochloride for 24 hrs. All

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three separated supernatant were fractionated by differential salt precipitation. Collagen type I molecules were precipitated with NaCl added to a final concentration of 2.6 M, pH 7.4, by stirring for 24 hrs and centrifuged at 15000g for 45 min. Collected collagen precipitates were dissolved in 2ml of 0.5 M acetic acid to make test sample stock solution. After preparation of sample stock solution, collagen type I standards were made with purified type I collagen from rat tail tendon (Sigma). 100 ml of 1 % Sirius Red (Direct Red 80) solution was prepared in 0.5 M acetic acid and was preserved for standard solution and test sample preparation.

Collagen Type I standard				
Final ppm Desired	Amt. 1000 ppm Stock (μl)	Amt. 0.5 M Acetic acid (μl)	Amt. of Sirius red (μl)	Total volume (μl)
5 ppm	10	190	1800	2000
10 ppm	20	180	1800	2000
20 ppm	40	160	1800	2000
30 ppm	60	140	1800	2000
40 ppm	80	120	1800	2000
50 ppm	100	100	1800	2000
Test Sample				
Amt. of extracted sample supernatant (μl)		Amt. of Sirius red (μl)		Total volume (μl)
200		1800		2000

The calibration graph was prepared using aliquots of collagen I standard solution. The concentration of collagen I was calculated in test samples with the help of equation obtained by the calibrated graph The sum of acid-soluble and enzyme collagen was defined as total collagen.

4.4.3 ALP Activity Estimation in Bone Samples

Phosphatases are enzymes which catalyse the splitting of a phosphate from mono-phosphoric esters. Alkaline phosphatase (ALP), a mixture of isoenzymes from liver, bone, intestine and placenta, has maximum enzyme activity at about pH 10.5. ALP measurements are of particular interest in the investigation of bone diseases. Paranitrophenyl phosphate, which is colourless, is hydrolysed by alkaline phosphatase at pH 10.5 and 37° C to form free paranitrophenol, which is yellow coloured. The addition of NaOH stops the enzyme activity and the final colour shows maximum absorbance at 410 nm. Process is same as in serum but for bone ALP estimation a homogenate was prepared for the assay.

Femur and Tibia were removed and cleaned of all adherent soft tissues, stored at -20° C until assayed. Samples of the bone (Femur and Tibia) were washed with physiological saline (0.9% NaCl) and blotted on filter paper. Each bone was homogenized in 2 ml of glycine buffer (pH 10.4) using a high-performance homogenizer. Homogenized samples were centrifuged for 20 min at 8000g and 4° C. The activity of ALP in supernatant of the femurs and tibia were determined using the same kit as for the total ALP in serum.

Working solution: Substrate powder (Disodium Paranitrophenyl phosphate) of each vial was dissolved in 10 ml buffer (ready to use and was supplied with kit). Solutions were colourless and stored in brown glass bottle at 2-8°C. These working solutions were stable for 45 days.

1 ml of working solution and 200 µl of sample supernatant were taken in a microcentrifuge tube. Before proceeding further, temperature controlled spectrophotometer (Cary) was calibrated at 405 nm wavelength to zero with the blank. Absorbance reading was done at 1 minute's time interval (0 min, 1min, 2 min, 3 min, 4 min.) at 37° C. ALP activity in IU/L is liberated milli moles of PNP per minute at 37^0C

incubation per liter sample. A_0 (absorbance at 0 min), A_1 (absorbance at 1 min), A_2 (absorbance at 2 min), A_3 (absorbance at 3 min), A_4 (absorbance at 4 min) were read against blank (distill water) and average change in absorbance per minute (Abs/min) was determined.

Calculation of PNP per minute in m.mol/L or ALP activity in IU/L in the test sample

$$= Abs / \min \times 275.7 \times tf$$

(Where the tf is temperature factor at 37° C, it is equal to 1 and multiplication factor is 2757 when 20 μl sample is taken but in this study 200 μl sample was taken, so multiplication factor in this case was 275.7)

CHAPTER-5

RESULTS AND DISCUSSIONS

RESULT AND DISCUSSION:

5.1.1:In Mineralogical Analysis, Bone Mineral Content (BMC), Bone Mineral Density (BMD), Calcium content, Phosphorous content and Carbon content of all three groups (control, HLS) were quantified.

A significant decrease has been found in the femur BMC of HLS group as compared to the control group (Table.5.1, fig.5..1,). BMC of Control group femur was 446. ±43.8 whereas BMC of femur in exposed group was 316.31±33.56 Similarly significant decrease in femur BMD level of HLS exposed group was found as compared to that of control group (Table.5.1, fig. 5,), BMD of femur of HLS exposed group was 733.17±58.82, and in the control group, it was 1011.33±73.8.

A similar trend was observed in the tibia. BMC and BMD of HLS exposed group was highly significant as compared with control group (Table5..1, fig.5.2.1 and fig. 5.2.2).). BMC of tibia of HLS exposed group was 220±27.75, and in control group it was 326.84±26.75. BMD of tibia of HLS exposed group was found as 543.2±45.72, and in control group it was 778.19±68.85.

This clearly indicates that HLS reduce the activity of mineralization (decrease in BMC) in induced osteoporotic rat bone. Results found not only less mineralization or less mineral precipitation in bone but also less mineral density also increased due to HLS exposure.

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Table : 5.1. Mean ± SD values obtained from Mineralogical Analysis

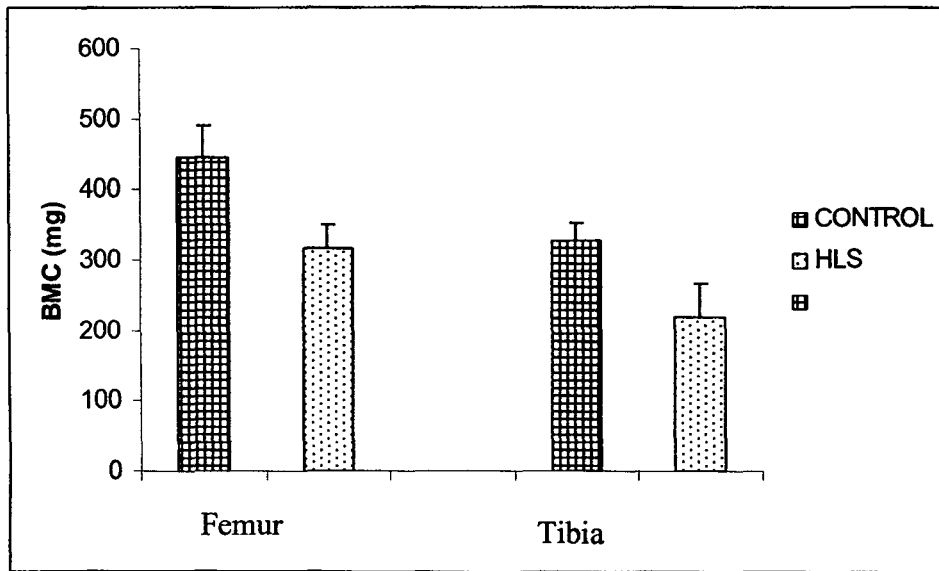|	Control		HLS	
	Femur	Tibia	Femur	Tibia
Fresh Bone Wt(gms)	846±45.75	621± 76.67	601± 57.62	418± 35.36
Bone Volume (μl)	0.441± 0.23	0.42± 0.15	0.431± 0.17.	0.405± 0.25
Dry Bone Wt(mg.)	678.4± 45.37	497.42 ±35.65	483.8± 58.8	335.65 ±73.54
BMC (mg)	446.1± 43.8	326.84 ±26.75	316.31 ±33.56	220± 27.75
BMD(mg)	1011.33 ±73.8	778.19 ±68.85	733.17 ±58.82	543.2 ±45.72
BOC (mg)	232.09±34'2	170.58±25	167.49±25.6	115.65±15.7

Figure:5.1 **Shows bone mineral content of femur and tibia of Control
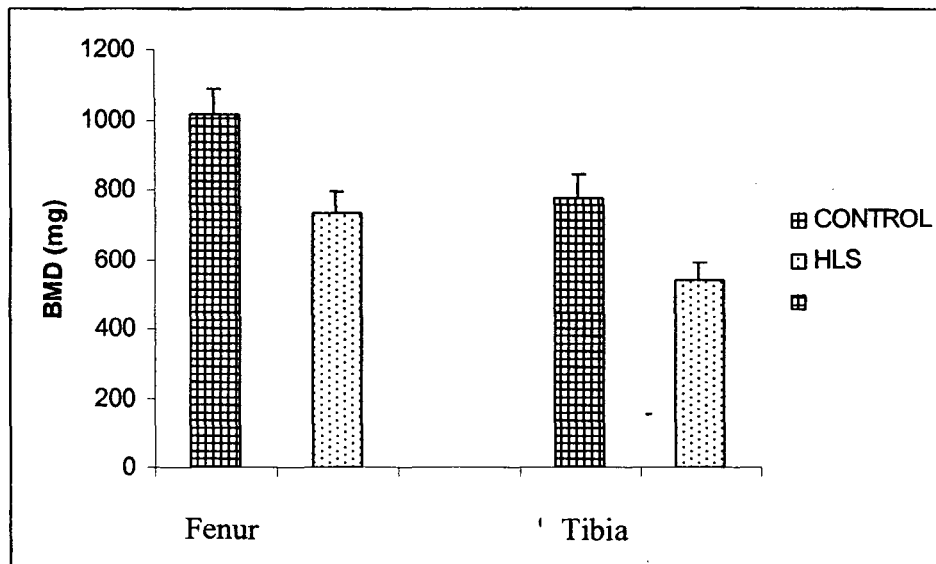and HLS**

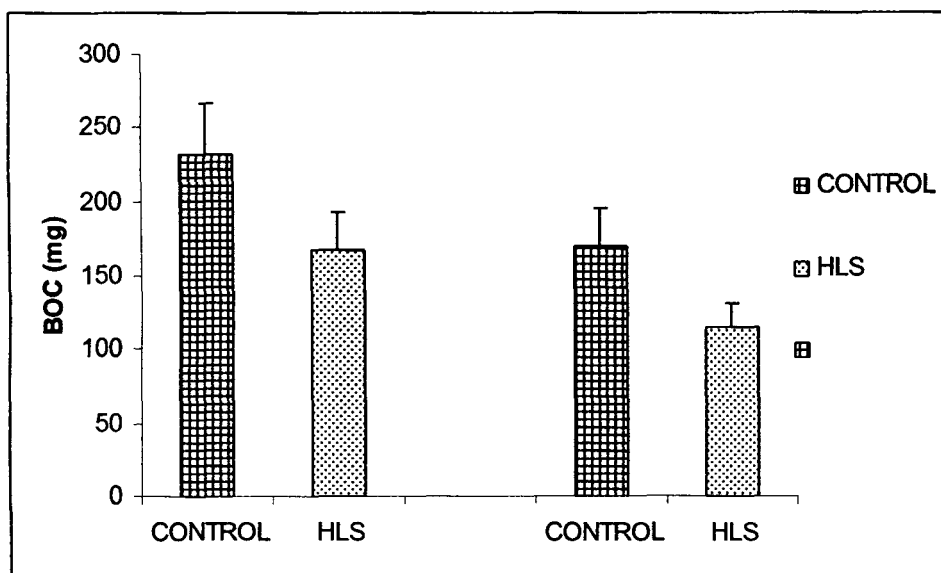Figure :5.2 **Shows bone mineral density of femur and tibia of control and HLS**

Figure : 5.3 Shows total bone mineral content of femur and tibia in control and HLS

Calcium and Phosphorous are the major constituents of bone minerals. Calcium was analysed in Flame Atomic Absorption Spectrometer (FAAS) and phosphorous was evaluated in spectrophotometer by colorometric method. . Whereas when compared with the control group it was found to be insignificant. Calcium content of femur in exposed group was 115±12.84 mg and Control group it was 128.44±18.51mg. Calcium content in tibia of Exposed group was 84.57 ± 3.26,: and in Control rat tibia was 86.69 ± 2.86.

Less Ca and P content shows the negative response of HLS exposure towards mineralization. Carbon content reflects organic content (Cellular components and extracellular macromolecules like Collagen).

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Table: 5.2

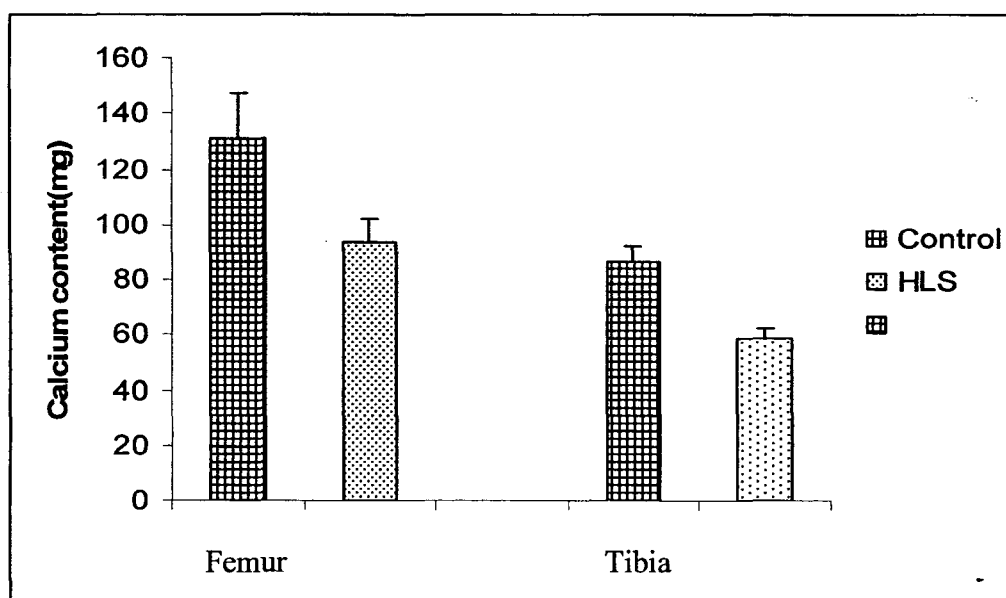|	Control		HLS	
	Femur	Tibia	Femur	Tibia
Calcium	131.12 ±16.25	86.55 ±5.78	93.56 ±8.53	58.73 ±3.5
Phosporous	112.61 ±2.5	73.12 ±3.65	80.31 ±4.6	49.34 ±3.6

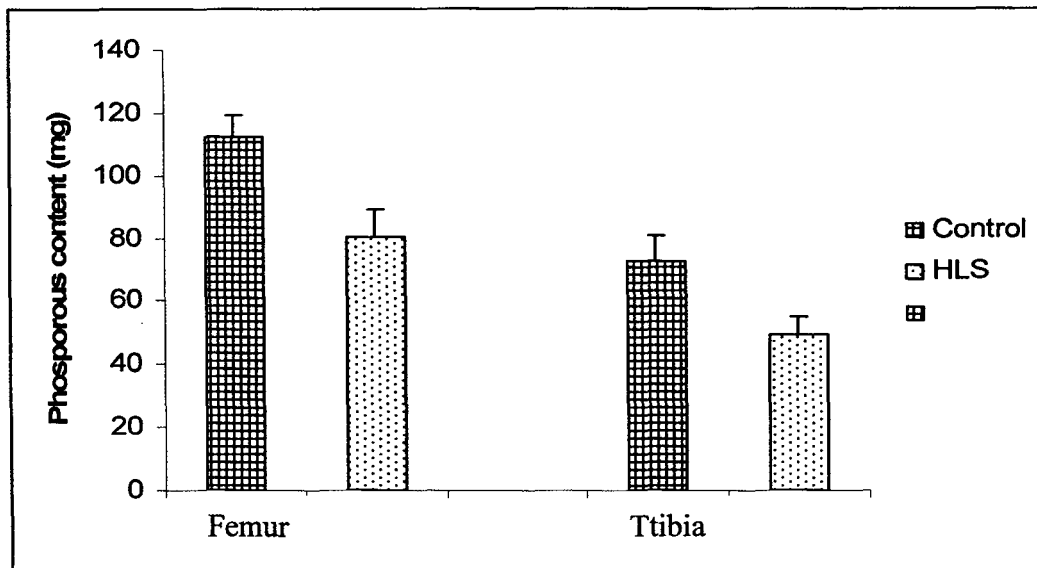figure : 5.4 Shows total calcium content of femur and tibia in control and HLS

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Figure : 5.5 **Shows total phosphorous content of femur and tibia in control and HLS**

An average decrease in BMD of 1-2 % per month in microgravity and simulated microgravity environments has been reported in the literature (27, 58). Previous studies have reported decreases in total BMD (1, 14, 21, 45), however they neither separated the different types of bone into compartments nor isolated a specific section of the bone. In this present study, we measured the BMD, BMC, calcium and Phosphorus content in both Femur and Tibia of control and HLS groups. We observed the significant decrease (P<0.05) in BMD, BMC, Ca and P content among control and Hind limb suspended (HLS).

5.2.3 Alkaline Phosphatase activity: ALP activity was measured in the Femur and Tibia of all groups.

The Alkaline Phosphatase is involved in mineralization process and good marker of osteoblast.The average values of ALP concentration in Femur and Tibia for different groups are mentioned in table (5.3) and shown graphically in fig (5.6)

Table 5.3 ALP activity (IU/L)

Groups	ALP in Femur (IU/l)	ALP in Tibia (IU/l)
Control	263±31.67	158±24.22
HLS	134±23.89	94±13.59
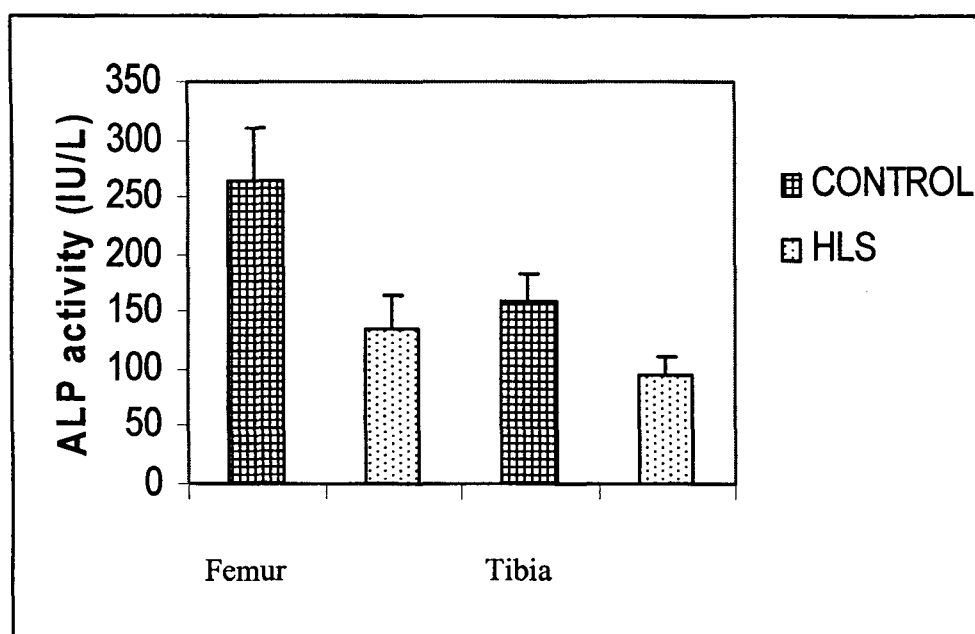
Figure: 5.6: Showing ALP activity in Femur and Tibia

There was significant decrease (P<0.05) in ALP activity in Femur and Tibia among Control and Hind Limb Suspended (HLS). Among the control and HLS, there was significant decrease (P<0.05) in ALP activity.

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The Alkaline Phosphatase is involved in mineralization process and good marker of osteoblast. Singnificant decrease (P<0.05) in ALP activity in Femur and Tibia of HLS compare to HLS indicates that mineralization and bone formation has been decreased due less proliferation of osteoblast in HLS.

5.242. Collagen I estimation in Femur and Tibia: Sirius red, a strong anionic dye, stains collagen by reacting via its sulphonic acid groups, with basic group present in collagen molecule. Collagen extracted with acetic acid and pepsin from bone samples of different groups were analysed.

Standard curve for type I collagen is shown in fig 5.5. The decrease of the absorbance of the dye solution reveals linearity with the amount of collagen.
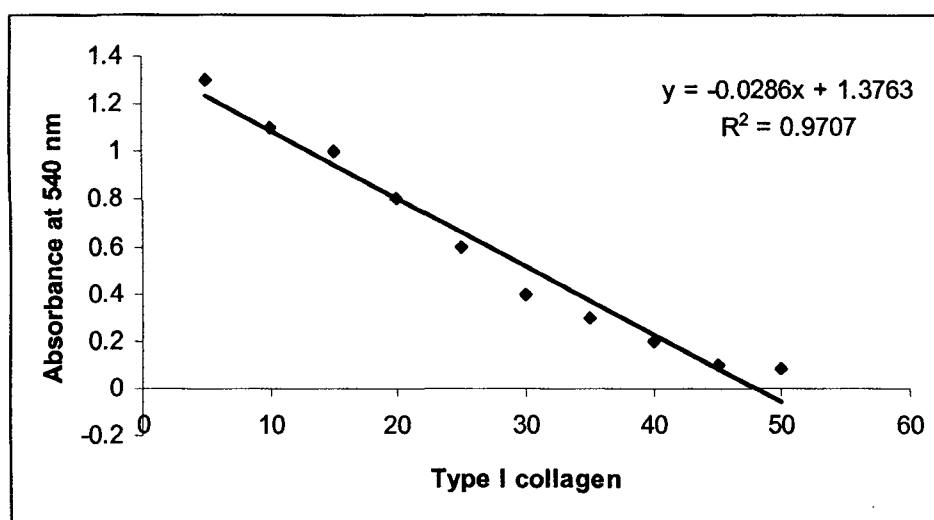

Fig5.7 Standard curve showing absorbance of Sirius red at 540 nm verses amount of type I collagen.

Table 5.4: Total Type I collagen in Femur and Tibia

GROUPS	Acetic digested type I collagen (μg/mg dry weight)		Pepsin digested type I collagen (μg/mg dry weight)		Total type I collagen (μg/mg dry weight)	
	Femur	Tibia	Femur	Tibia	Femur	Tibia
CONTROL	942.97± 25.17	731.2± 45.78	1395.33± 22.65	1066.96± 17.56	2338.3± 45.54	1798.16± 35.23
HLS	667.64± 17.58	495.08± 34.6	995.17± 56.28	718.29± 35.6	1662.81± 37.27	1213.37± 47.15

The average values of total concentration of collagen I (acetic acid digested collagen + pepsin digested collagen) in Femur and Tibia for different groups are mentioned in table (5.3) and shown graphically in fig (5.6) and (5.7).
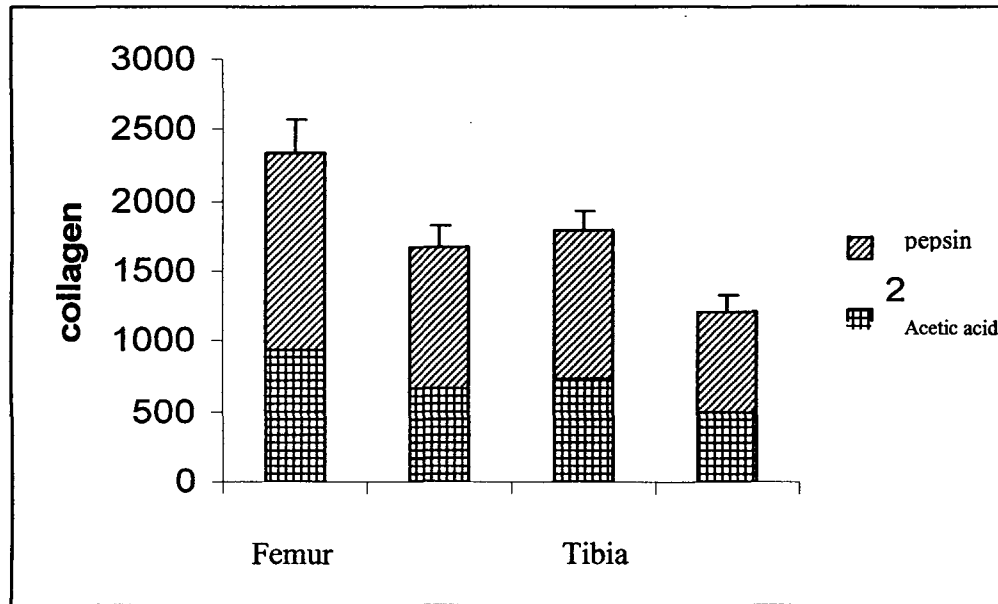

Figure 5.8: Showing Type I collagen (μg/mg of dry weight) in Femur and Tibia.

There was significant decrease (P<0.05) in Type I collagen in Femur and Tibia of control compare to Control.

Although Alkaline Phosphatase and Osteocalcin are two primary markers for bone formation, use of Collagen I may be reliable marker for bone formation, because biosynthesis of collagen I is also depend on osteoblast like ALP and Osteocalcin.Due to cross-linking property , collagen has dynamic role in mineralization, regulating the size, shape, and site of mineral crystal within the fibrils(Yamauchi,1996) .

Significant decrease in Collagen I among HLS and control indicate that bone mineralization has been decrease due hind limb suspension.

5.1.5 The Histological Analysis

The SEM of eight samples of (1) Cancellous bone of femur head in control (2) Cancellous bone of femur head in HLS (3) diaphysis bone of femur in control(4) diahysis bone of femur in HLS (5) proximal part of tibia (6)proximal part of tibia in control (7) diaphysis bone of tibia in control and(8) diaphysis bone of tibia in HLS were carried out to investigate how the structural morphology of the bone would change.Theses sample for surface analysis were prepared by cross-sectional bisecting the bone sample at required portion. Using vacuum coating technique, gold coating was done on the samples. The scanning electron micrographs were taken using' low vacuum SEM' *Leo 435 VP* .
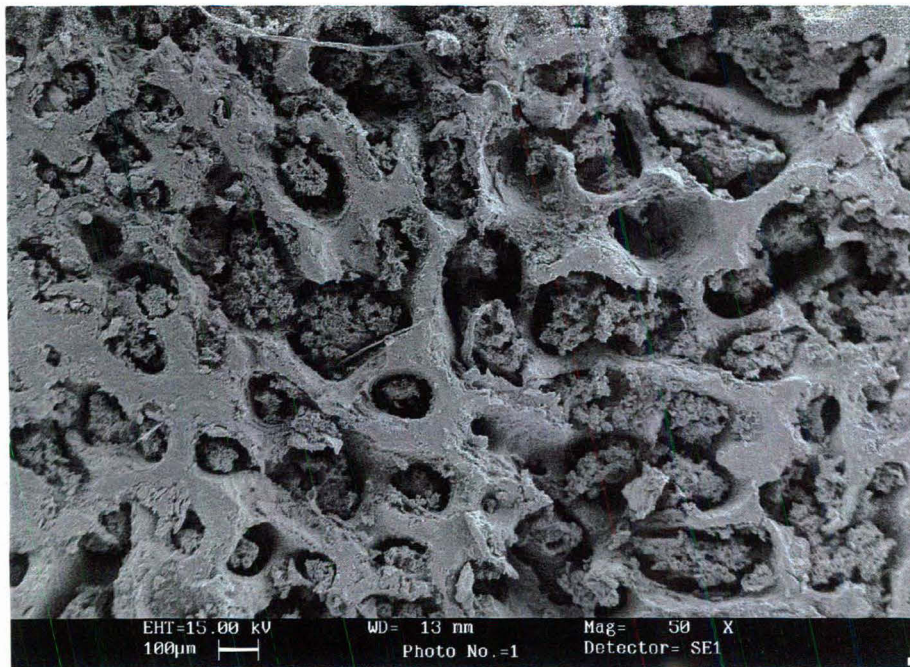
.

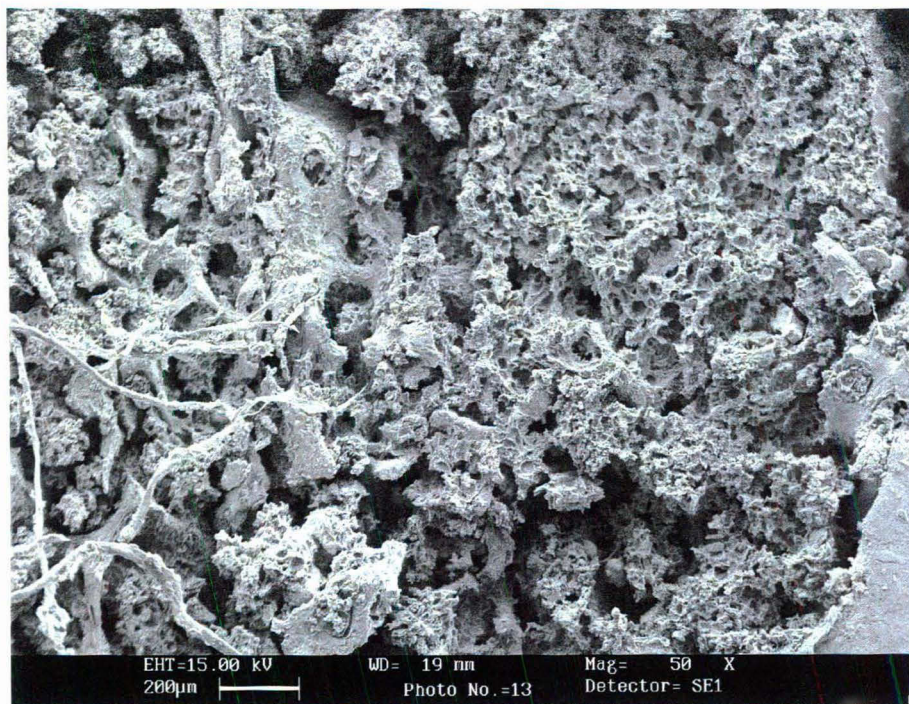Figure 5.9 Show the SEM images of Cancellous bone of femur head in control

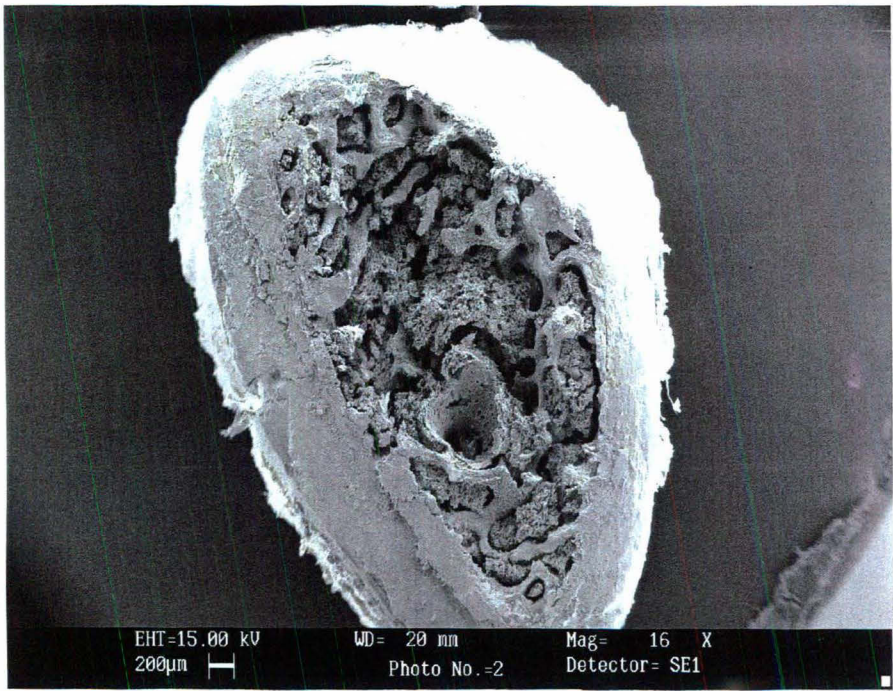Figure 5.10 Show the SEM images of Cancellous bone of femur head in HLS

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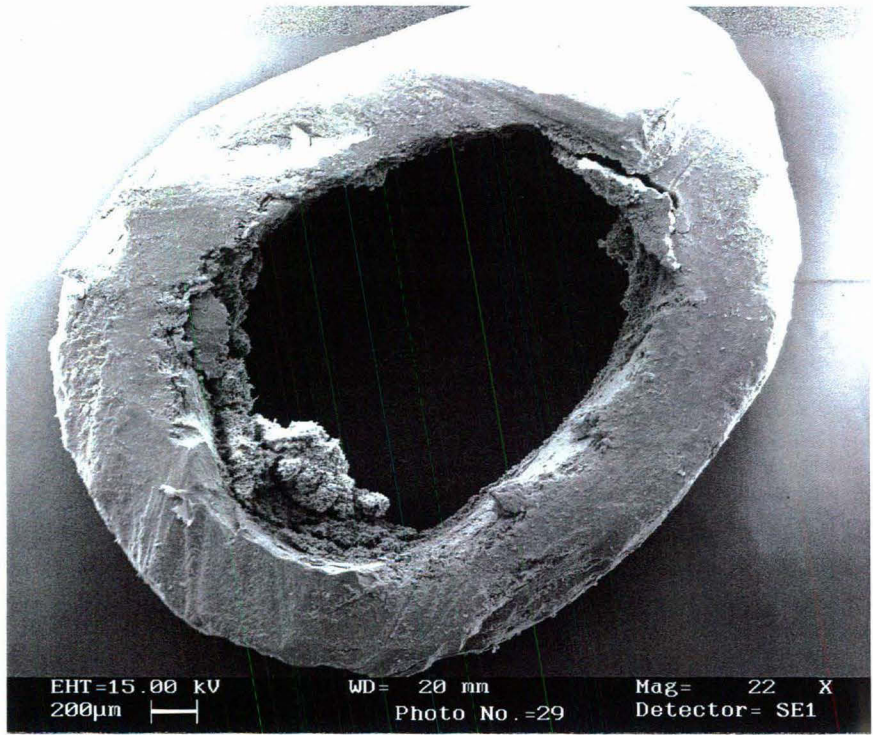FIGURE 5.11. SEM image of diaphysis bone of femur in control

FIGURE 5.12 SEM image of diahysis bone of femur in HLS
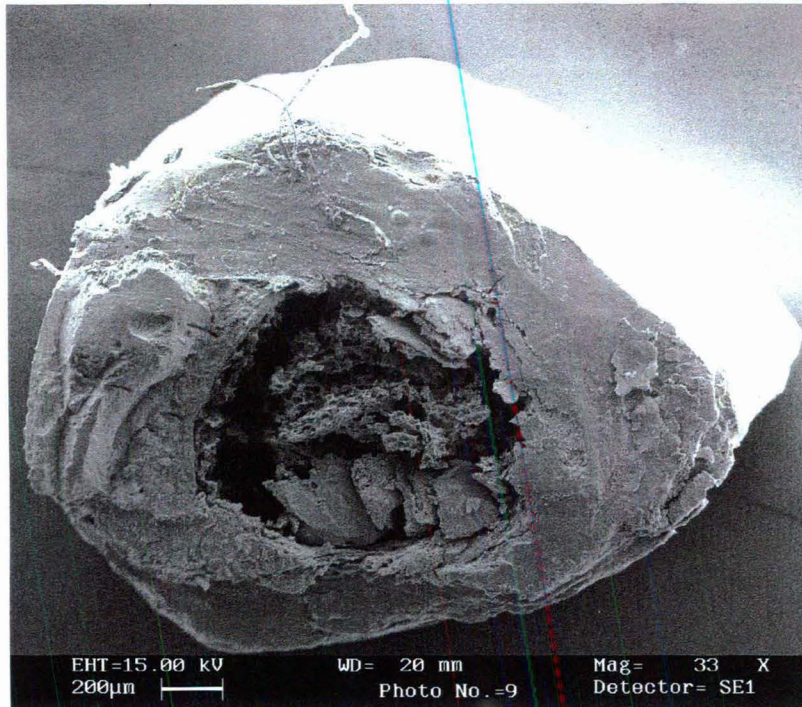
figure : 5.13Shows the SEM image of proximal part of tibia
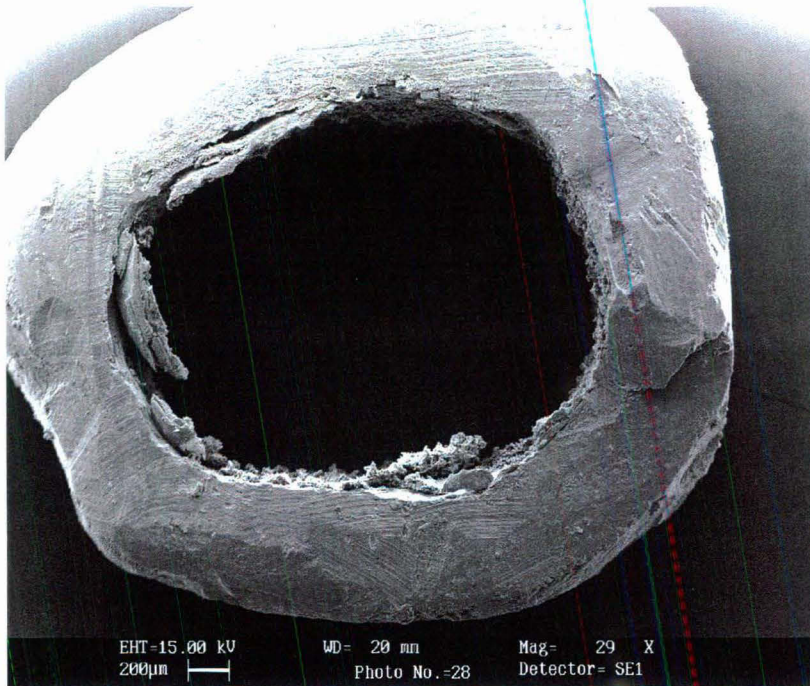
figure : 5.14Shows the SEM image of proximal part of tibia
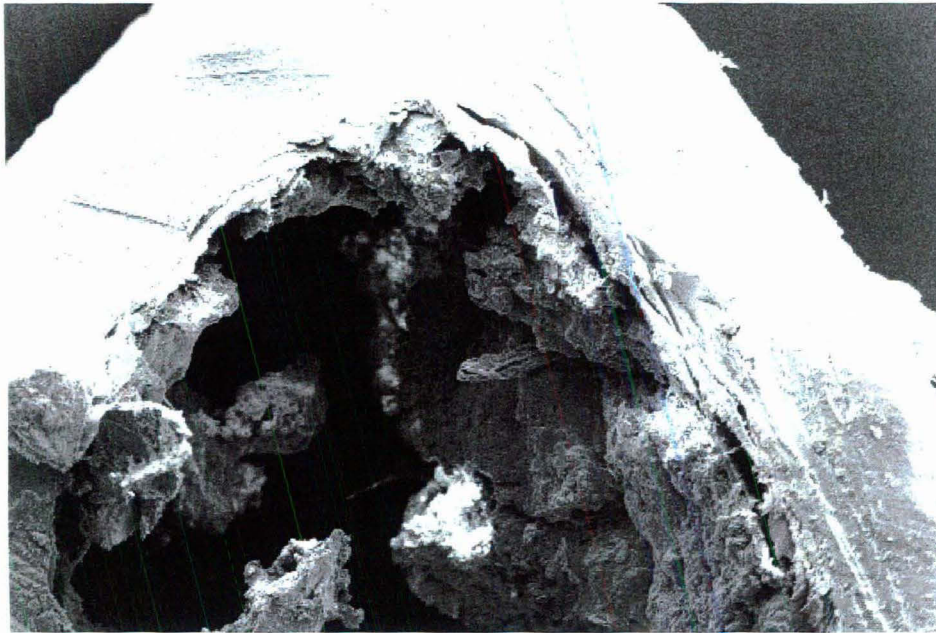
Figure :5.15 SEM image of tibia
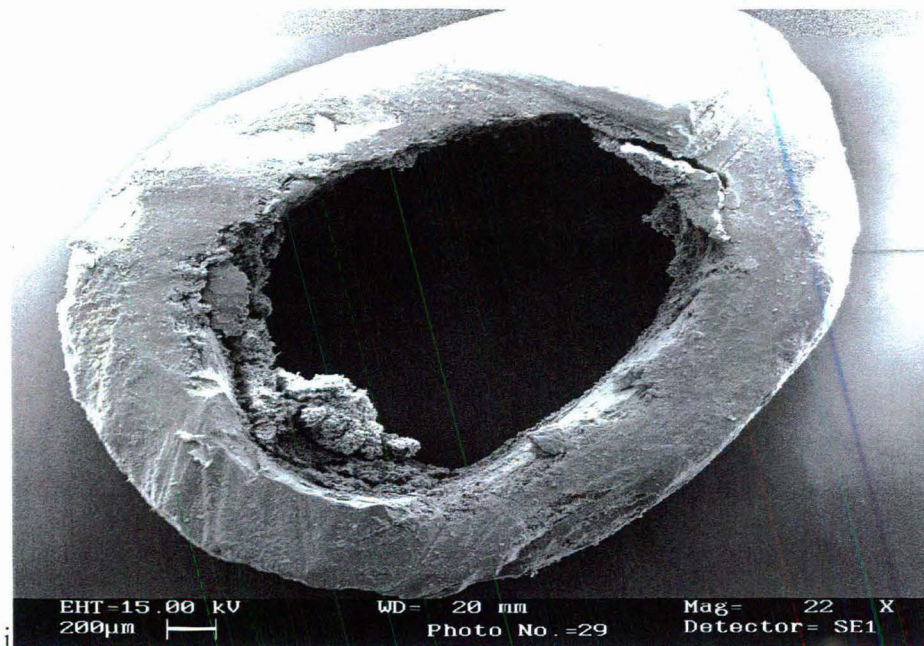
Figure : 5.16 SEM image of tibia in HLS

Light micrograph of SEM **analysis** showed the control and HLSgroup bone marrow of same place in femur diaphysis are shown in the figure (H.E. 40X). Bone cells are heavily populated in control group whereas in HLS condition they were less populated and osteoclast (multinucleated cells) like cells were clumped together. Less osteoblasts than osteoclasts were found in HLS exposed group as compared to the control group

CHAPTER-6

CONCLUSIONS

Conclusion:

In this work we have done quantitative analysis of the bone loss of the rats in control and in HLS condition. A comprehensive study has been carried out on BMC, BMD, and BOC of the bones (femur and tibia) of the rats of which one groups were in control and other exposed to simulated microgravity . Biochemical analysis , Collagen estimation and ALP estimation have also been carried out. Further histological study of the transverse-section of the bone at different region have been carried out by SEM (Scanning Electron Microscope) analysis. The details of our finding are given in the followings.

- BMD,BMC,Ca and P content in femur and tibia was significantly less in Femur and tibia of HLS as compare to those of control groups

- Alkaline phosphatase activity was significantly less in Femur and Tibia of HLS as compare to those of control groups. It indicates that osteoblast proliferation may decrease due to long term space flight.

- Collagen I concentration was also significantly decrease in Femur and Tibia of HLS as compare to those of control groups.

- Cortical thickness in femur and tibia was also less in HLS as compare to control.

Above result, indicate that long term space flight has severe effect and effective countermeasure is required to overcome this problem.

CHAPTER-7

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