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In vitro biocompatibility study to determine the synergistic effect of components for an artificial bone graft material

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ABSTRACT

Title of Thesis:

In Vitro Biocompatibility Study To Determine The Synergistic Effect of Components for An Artificial Bone Graft Material

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A composite bone graft material which can induce or promote new bone growth has potential orthopaedic applications in the repair of bone defects or the stabilization of surgically implanted devices. This material should be biocompatible, have good handling properties, promote osteoinduction and/or osteoconduction and have a good shelf life. Demineralized bone matrix and bone morphogenetic protein have been demonstrated to promote osteoinduction *in vitro* and *in vivo*.

This *in vitro* study examined the inductive ability of an osteoinductive protein, bone morphogenetic protein, when absorbed on non-active devitalized bone matrix. In culture, fetal rat muscle was grown on powdered rabbit demineralized bone matrix (positive control), devitalized bone matrix (negative control) and devitalized bone matrix plus bone morphogenetic protein to determine the differentiation of the mesenchymal cells, chondrogenesis. This modified tissue culture model showed chondrogenesis of the cells grown on the demineralized bone matrix, no chondrogenesis with devitalized bone matrix and unexpectedly, no enhancement of

the chondrogeneis with the osteoinductive factor added to the devitalized bone matrix.

IN VITRO BIOCOMPATIBILITY STUDY TO DETERMINE THE SYNERGISTIC EFFECT OF COMPONENTS FOR AN ARTIFICIAL BONE GRAFT MATERIAL

by
Levelle Elise Burr Alexander

Thesis submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Engineering.

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APPROVAL SHEET

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Dedicated, with love

to my husband, Jesse N. Alexander III,

and

in memory of my grandmother, Mable O. Franklin Wheeler.

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INTRODUCTION

The medical profession has used various types of materials to repair or replace bone segments which have been damaged or surgically removed. These materials fall into such categories as banked bone, bone grafts, carbon based, ceramics, collagen, glasses, metal alloys, metals, polymers and composites. Each of these materials possess their own mechanical properties, [see Figure 1 and Table 1]. The most common material used for "grafting" is autogenous bone grafts where the bone is transferred from one bone site to another site in the same individual. Unfortunately, autogeneic grafting is not always possible, therefore an alternative is allogeneic grafting (bone received from another individual). Allogeneic bone is not transplanted *in situ*, but is immediately "banked" (frozen) for later use. Significant problems are assoicated with the use of banked bone. Many problems hinder the success of the transplant. Whenever any foreign tissue, including allogeneic bone, is implanted or transplanted into the body, there is usually a risk for local tissue infection and transmittal of systemic viral infections, for example acquired immune deficiancy syndrome (AIDS) or hepatitis and also stimulation of the immune response system. This can result in the gradual rejection of the implanted or transplanted material.

A foreign material that will not elicit a foreign body or immune rejection is called a biocompatible material and is sometimes referred to as a biomaterial. As noted above, a number of biomaterials have been tried as bone replacements.

Those materials which have the ability to repair damaged segments by bony ingrowth can have osteoinductive or osteoconductive properties, as opposed to one that is osteogenic. An osteogenic material is one that contains the necessary bone cells to allow the development of new bone through mineralization of the matrix that surrounds the new cells. An osteoinductive material will induce the conversion

of the undifferentiated mesenchymal cells to cartilage cells, which are the precursor for new bone cells. An osteoconductive material will allow the conduction of new bone cells from an osseous site to a non-osseous site by acting as a trellis for the incorporation of the new bone along the material's surface. To understand the cellular processes for a successful bone graft, the mechanisms for bone growth, bone remodeling, and fracture healing are presented.

BONE

Bone is a living, specialized connective tissue in the body that undergoes five major processes: endochondral ossification (growth), modeling, remodeling, fracture repair, and ion storage for bone-blood exchanges. All of these processes help to maintain the dynamics of the mechanical, hormonal and structural demands placed on the localized bone regions in humans. On a structural level, bone consists of ~85% extracellular and ~15% intracellular matrix (by dry weight) depending upon the age, bone type and animal species, [see Figure 2]. These matrices have been determined to contain an inorganic (mineral) and organic phase. For the mineral phase, the major component (60% by weight) is hydroxyapatite, $Ca_{10}(PO)_6(OH)_2$, a source of calcium ions. For the organic phase, the major component (88-90% by weight) is collagen, with a small percentage of non-collagenous proteins. The bone tissue can be arranged in bundles, woven or fine fibers. Two types of bones exists in humans: compact, cortical bone and spongy, trabecular (cancellous) bone. On a cellular level, many cells types have been identified: mesenchymal cells, chondrocytes, cartilage, osteoblasts, osteoclasts, osteocytes and mature bone cells.

Since human bones are constantly undergoing cellular change, it is difficult to measure many of the mechanical properties of live bone, hard tissue, in terms of a consistant numerical value. The values of the mechanical testing will vary due to the composite structure of the bone (cortical verus cancellous), age of the bone, excision and handling procedures used to acquire the bone specimen (homovital or homostatic), and the procedures used to test the bone specimen. As demonstrated in Table 1, bone is more ductile than some plastics and more flexible than most metals or ceramics. Applying the information known about the mechanical, cellular and hormonal properties of bone, researchers have been investigating the possibility

of using excised bone as a "natural" biomaterial. This "natural" biomaterial should ideally restore as much of the regional mechanical and cellular demands.

A. Long Bone Growth - Modeling Process

From birth to the end of adolescence, the prenatal bone cells forming the analage cartilage in human long bones are converted into mature bone cells by a process called enchondral or endochrondral ossification. This growth conversion takes place at two sites in long bones. The first site is in the shaft or stem region of the bone where the growth is marked by a radially outward pattern from the prenatal bone cell towards the metaphysis, [see Figure 3(a) and 3(b)]. In the femur, the metaphysis is called the femoral neck. The second site is at the two end regions of the long bone called the epiphysis where a similar radially outward growth process takes place from a prenatal bone cell towards the metaphysis until the two sites for growth meet at the region called the epiphyseal growth plate, [see Figure 3(a) and 3(b)]. In the femur the epiphysis is called the femoral head.

The epiphyseal or cartilage endochondral growth plate consists of six zones [Matthews (1980)]: the resting or reserve cartilage zone, the proliferation zone, the enlarging or matrix synthesis zone, the cell hypertrophy zone, the provisional calcification zone and the ossification zone, [see Figure 4(a)-(c)]. The reserve cartilage zone is located adjacent to the articular cartilage marked by small chondrocytes oriented in a random order. In the proliferation zone, the disc shaped chondrocytes increase in size with their processes extended out into its surrounding matrix and their orientation begins to become ordered in parallel columns. The matrix synthesis zone is marked by the enlarged chondrocytes with their distinctive ball shaped processes extending further into the newly synthesized cartilagenous matrix. This cartilagenous matrix separates the "well ordered" parallel columns of the enlarged chondrocytes. In the cell hypertrophy zone, additional cellular definition of the chondrocytes are observed and the histological tissue fixation technique used will determine the cellular morphology. The provisional

calcification zone is marked by the irregularly thickened cartilage matrix that surrounds the parallel columns of the enlarged chondrocytes. The ossification zone is marked by the calcification of the cartilage matrix.

The outward growth from the prenatal bone cell towards the metaphysis is controlled by the rate of calcification in the ossification zone. This allows for the lengthening of long bones during post natal development, thus is one method by which long bones transform in length. A second method long bones transform is through the width widening during post natal development. This process is achieved through the slow removal of "old" bone cells from the endosteal region, the inner most surface of the cortex, and the slightly faster depositing of "new" bone cells on the periosteal region, the outer most surface of the cortex, [see Figure 5]. A third method by which bone is remodeled is the continuous deposition and resorption of bone without the changing of the bones overall structure. It should be noted that any malfunction or disruption in the deposition or resorption process can effect the ideal mechanical properties of bone. The feedback mechanism by which bone responds to mechanical stress is poorly understood; but it has been postulated by some researchers to be related to the stress generated electrical properties of bone [Trehane (1981)].

In order to maintain the structural and mechanical requirements imposed on bone at various locations in the human body, there exists two forms of bone: (1) traebacular or spongy cancellous bone and (2) cortical or dense bone. For example the composition of bone at the femoral head (the epiphysis) is largely spongy and compact or cortical bone is found in the mid-shaft of the femur, [see Figure 5]. In both bone forms, there are many types of skeletal tissue cells that contribute to the growth, repair and maintenance on a cellular level:

1. Undifferentiated mesenchymal cells which are sometimes referred to as osteoprogenitor cells whose function has been postulated to be responsible for the replication of daughter cells that later transform into functioning chondro-osseous cells, [see Figure 6].

2. Chondroblast cells which are the precursor to chondrocytes are found to be located in the pericanalicular tissues of the cartilage canals. These cells are found along the epiphyseal perichondrium and along the zone of Ranvier. Once the cells have undergone cellular division a lacuna or pocket is formed with a surrounding matrix, [see Figure 6].

3. Chondrocytes cells are located in the lacunae (pockets) marked by the cells enclosure in an extracellular matrix called chondroid. The chondrocytes processes extend out into this matrix. The function of these cells are to maintain the surrounding matrix during enchondral development prior to the ossification of the cartilage matrix, [see Figure 6].

4. Osteoblast cells are responsible for producing a protein called osteoid. These "polarized" cells tend to align themselves such that the cell nucleus is directed away from the adjacent bone surface while the branched and unbranched processes extend not only into the chondroid matrix but also into the canaliculi of the bone. It has been postulated that stimulation of these cells result in the deposition of "new" bone matrix and it is through their cell processes that the transport of important chemical nutrients and waste are achieved, [see Figure 6].

5. Osteocyte cells are mature cells in the lacunae that are oriented in parallel columns called lamellae. Surrounding the lacunae is a pericellular space containing proteins, lipids, collagen fibrils and protein polysaccharides which separate these cells from the mineralized osteoid. These cells have been postulated to stimulate the resorption of "old" bone cells through osteocytic osteolysis in the bone remodeling process, [see Figure 6].

6. Osteoclast cells are multi-nucleated cell with an increased plasma membrane surface. According to many researchers, it is the high enzymatic levels of these cells that lead to the breakdown of the "old" bone; this causes the formation of Howship's lacunae or cutting cones, [see Figure 6].

The interaction of all these cells lead to the cascade of endochondral bone differentiation. According to Sampath and Reddi this cascade progresses in the following steps [Sampath (1981)]: mesenchymal cell chemotaxis, cell proliferation, cell differentiation, hypertrophy of the chondrocytes, vascular invasion, calcification of the cartilage matrix, formation and remodeling of bone, and differentiation of hematopoietic bone marrow within the newly formed bone, [see Figure 7].

B. Long Bone Remodeling

1. Natural Remodeling Processes

a. Wolff's Law

In 1892 Wolff presented the law of bone transformation, referred to as Wolff's Law, which states in part "...every change in the function of bone is followed by a certain definite change in the internal architecture and external conformation in accordance with mathematical laws" [Trehane (1981)]. This bone transformation can result from internal hormonal stimulation, external load/stress stimuli or genetic coding. Currently this law has become synonymous with explaining the observations made from bone's adaptation to external stresses. Bone has been found to respond to stresses by net bone deposition (growth) or net bone resorption (removal) referred to as piezotropism. This phenomenon describes a dynamic adaptation response.

b. Cellular Remodeling Model

In 1984, Parfitt presented a cellular model for the cyclical remodeling of adult bones consisting of five stages [Parfitt (1984)]: quiescence, activation, resorption, reversal and formation, [see Figure 8]. The bone cells located on the inner region of bone nearest to the marrow cavity form a five layer quiescence surface that must be activated by a change in the local load or stress in order for the remodeling process to initiate.

As the activation progresses to the resorption stage, the osteoclastic precursor cells penetrate the unmineralized connective tissue layer (thin flat lining cells) of the quiescence surface. This brings the precursor cells closer to the storage site of the bone minerals located in the bone matrix. It should be noted that the control of the shape, size or rate of the conversion is unknown. The termination of

this process however has been traced to the release of a suppressor agent from the osteocytes and/or the lining cells. This step occurs in human adults approximately every ten seconds.

During the resorption stage, the newly formed osteoclastic cells begin to locally resorb the "old" bone matrix forming an erosion cavity called a Howship's lacunae in cancellous bone and a cutting cone in cortical bone. As the surface is eroded, the osteoclastic cells attach to the erosion site in the bone cavity through a temporary seal. Complex chemical interactions are responsible for the temporary attachment and resorption ability of the osteoclasts. It is proposed that in this stage, specialized growth factors are released which allow the transition from the resorption stage to the formation stage. It is this transition which initiates the bone deposition process.

The reversal stage is marked by the surface of this roughened cavity becoming smooth then lined with a cement-like substance that contains highly mineralized, collagen deficient bone matrix. The filling of this cavity prepares the site for the next step, the formation stage. This cement lining has been used as a marker since it can be detected under a microscope. The growth factors released for the transition from the resorption stage to the formation stage enable the differentiation and proliferation of the osteoblastic precursor cells.

As a result, the newly formed osteoblasts selectively accumulate into a uniform, single layer with polar alignment in this cavity. In several days, the osteoblasts rapidly form the unmineralized matrix called the osteoid. This is followed by a rapid surge in the mineralization of the matrix. The mineralization process will slow down once the matrix has assume a width of approximately 20 microns. The step is completed by the erosion cavity being replaced by young living bone cells.

c. Fracture Healing Process

Fractures in mammalian bone skeletal structures are due to the upper limit of the strain energy being exceeded. As a response to the fracture, localized systemic and tissue responses are triggered to ultimately restore the biomechanical integrity and metabolic (cellular) requirements. The amount of time necessary for "complete" repair is dependent on several factors: size and shape of fracture line, mechanical loads placed on fractured region and age of the bone.

Some localized effects from a fracture are hemorrhaging, disruption of local blood supply, anoxia and/or local cell death (necrosis). This leads to the initiation of the following tissue responses similiar to the cellular responses observed histologically in "normal" bone remodeling, [see Figure 9, Simmons (1985)]:

> Stage 1: Hematoma Formation. Caused by an aseptic inflammatory reaction.
>
> Stage 2: Callus and Temporary Vascular Formation. Proliferation and differentiation of osteoprogenitor cells into functionalizing fibroblastic, chondrogenic and/or osteogenic cells. This is acheived within the cambium layer of the periostem and at the endosteum. As the osteoprogenitor cells begin proliferation, a thickening in the local periosteum is apparent. When the internal micromotion of the bone fragments increase, the osteoprogenitor cells differentiate into hyaline cartilage and/or a fibrocartilagenous callus. The process of callus formation is referred to as callus chondrification. This helps to stablize the fracture site by joining the fractured bone ends until new bone is deposited to restore the mechanical integrity. Completion of callus chondrification is followed by callus calcification. This calcified callus also provides a temporary bed for the blood supply. The local

blood supply provides nutrients and removes metabolic waste products during the fracture remodeling stage.

Stage 3: Resorption of Necrotic Tissue. This occurs simultaneously with the latter phase (callus formation). The necrotic (dead) tissue is removed from the fracture site to allow for the subsequent deposition of "new" bone.

Stage 4: Remodeling Phase. It is postulated that as the resorption of the necrotic bone takes place, a collagenase-resistant glycoprotein links the cellular interaction between the necrotic bone matrix and the new bone cells. This glycoprotein was named bone morphogenetic protein (BMP) by Urist in 1965. The net result is local endochondral substitution by fibrous, lamellae bone.

Stage 5: Revascularization. Restoration of the local blood supply at the repaired fracture site.

Exceptions to the callus formation during the fracture healing process have been observed for several conditions: fracture healing that is aided by a compression plate where a parallel deposition of new bone transverses the fracture site by "osteoclastic cutting cones" and fracture healing in the skull where the deposition of new bone is regulated by the modulation of periosteal progenitor cells during the differentiation into osteoblastic cell.

2. Medical Alternatives for Long Bone Repair

Medical alternatives are available to enhance the local bone growth at a damaged bone site if natural remodelling and fracture healing processes can not repair the damage. These alternatives are useful for conditions such as:

1. a massive fracture which would normally lead to a non-union;
2. bone segments that are surgically removed due to tumors, cysts or injury;
3. painful joints that need to be fused;
4. the restoration of cosmetic appearance or structural deficiencies of a bony region.

A non-union fracture results from the fractured bone ends being too far away from each other to allow the formation of a callus which stabilizes the damaged site until the healing process is complete.

a. Bone Grafts and Implants

Repair alternatives are usually acheived through surgical transplantation or implantation of a bone graft, bone implant or bone implant/graft substitute. The term bone transplantation refers to the surgical procedure where the hard bone tissue is transferred on a vascular bed. An example is freshly excised, homovital (live) bone that provides an autograft or allograft. A bone implantation refers to the surgical procedure where the transferred bone is non-viable (contains dead bone cells with mineral, collagen and some active proteins). An example is banked bone that has been frozen or freeze-dried and processed; thus it is homostatic (dead) bone.

There are several sources for the bone graft (freshly excised) or bone implant (banked):

1. autogeneic bone graft or implant, if the source or donor is genetically identical (from the same individual as the recepient, but transferred to a different site);

2. allogeneic bone graft or implant, if the source or donor is geneticallly non-identical (from the same species but a different individual than the recipient);

3. synogeneic bone graft or implant, if the source or donor is genetically similiar (from the same species but a different individual than the recepient), for example identical twins;

4. xenogenic bone graft or implant, if the source or donor is genetically non-identical (from a different species than the recepient);

5. synthetic bone graft or implant, if a synthetic material is used as a substitute for the bone.

A bone graft/implant substitute currently refers to a composite grafting material consisting of frozen or freeze-dried, banked allogeneic bone along with autogeneous bone marrow from cancellous bone. However in the future, synthetic materials may be used.

For many years there has been a search for suitable synthetic materials to aid in the repair of bone. These materials have a wide range in their physical and mechanical properties, for example metals and metal alloys, glasses, ceramics and polymers (natural and synthetic). Depending upon the material and design, this synthetic graft/implant may acts as a permanent implant or as a means to promote new bone ingrowth. Some of these materials may gradually resorb as the bone ingrowth process proceeds.

The type of bone used for the graft or implant is dependent upon the location of the damaged bone region (weight bearing verses non-weight bearing), bone osteogenic function, size and shape of the damaged region, availability of the patient's own bone as an autogeneic bone graft, and the availability of suitable banked bone. Spongy cancellous bone, usually from the iliac crest, or chunked cortical bone is used to repair small segments or cavities. Compact cortical bone is used to repair those regions that have the regional demands for good mechanical strength. Cortical bone is used in the fusion of the transverse processes of the spine or in the regions that require the grafting material to be shaped to a specific size. Usually the choice of bone type is cortical bone due to it's initial mechanical strength at the time of transplantation or implantation, the enhanced osteogenic properties, and the relatively large size which can be shaped to the desired recepient size. Note that in the subsequent discussion of bone repair by bone grafts or implants, the broad term "bone graft" or "graft" will be used with qualificiations, if necesary, as to whether the material is homovital or homostatic.

After the bone graft has been transferred to the recepient site there are three possible consquences [Prolo (1985)]. The first is that the graft can acheive the mechanical, cellular and cosmetic characteristics of the adjacent bone. The second is that the graft can become partially or completely resorbed. The resorption can consequently cause local disfigurement or instability which can also affect the function of local internal organs. Finally, the graft can become sesquestrated, encapsulated and treated as a foreign body by the host immune response system.

In 1978 Burchardt and Enneking described some of the similiarities and differences that they observed in cancellous and cortical bone grafts using a canine model to simulate human segmental grafts [Prolo (1985)]:

1. Both bone types within the first two weeks of implantation showed similiar signs of coagulated blood around the graft, an initial immune response resulting in the formation of fibrous granulation tissue, an increase in the osteoclastic activity along with osteocytis autolysis.

2. Differences in the two were evident after two weeks by their rates of revascularization, mechanism and completeness of repair and the mechanical properties of the graft itself. For example, cancellous bone will begin to revascularize within hours (versus days for cortical bone) resulting in completion within two weeks; and can achieve close to 100% remodelling.

The results of analyzing animal models, clinical experiments and hard tissue bone grafts from cadavers through mechanical, biological and histological techniques have given invaluable information for evaluating the extent of bone graft incorporation. The term incorporation refers to the envelopment and interdigitation (resorption and deposition of bone) of the donated hard tissue in the recipient bed. It has been determined that cancellous bone is better than cortical bone in the degree of incorporation. Preferrably the bone should be an autogeneic graft instead of an allograft. The following is a summarized list of the donor bone for grafting in decreasing order of repair results [Urist (1980), Prolo (1985)]: fresh autogeneic bone, frozen autogeneic bone, autolyzed antigen-extracted allogeneic (AAA) bone, freeze-dried or frozen allogeneic bone, irradiation-sterilized or chemically sterilized allogeneic bone, xenogeneic bone (no longer used in humans), vascularized grafts (through advances in microsurgery), "composite" bone grafts (banked bone with marrow from cancellous autogeneic bone), and synthetic bone grafts (especially in the applications of "ceramics"). The latter three alternatives are still in the preliminary stages of clinical applications.

The preparation time and procedure of autogeneic and allogeneic donor bone is important to the overall success of this "natural" biomaterial's effectiveness as a bone graft. For surgical procedures of homovital (fresh) autogeneic grafts, it is necessary for the recipient site to be prepared for the donor bone by removing or cleaning the damaged bone. The donor bone should be excised, trimmed to the proper size and immediately transferred to the donor site ideally without any exposure to external solutions.

For homostatic (banked) allogeneic grafts the donated bone is usually excised from amputees, cadavers or from the patient's ribs. Once the donor bone is excised, the grafting material must be cleaned of any soft tissues under sterile conditions and if necessary, treated to sterilize or to reduce the antigenicity. The bone grafting material is sealed in double packaging, temporarily stored while the bone tissue is cultured and labelled, then permanently stored at -70^{O}C. A collection time of greater than twelve hours for excised cadaver bone can negatively effect the activity of some of the enzymes that will survive the banking process. Incomplete removal of soft tissue has been postulated to contribute significantly to an increase in the antigenicity, immune response. Treatment of donor bone with certain chemicals, for example strong acids, or high dose radiation, for example gamma irradiation above 20 mrad, can destroy and denature critical proteins and enzymes. Treatment of donor bone with immediate freeze-drying or "chemicals" to cause autolytic digestion of osteocytes have been found to enhance success (reduce rejection and increase incorporation). Temperatures above minus 70^{O}C should be avoided during storage since higher temperatures of 0 to 30^{O}C will denature a critical protein called bone morphogenetic protein (BMP).

When the bone graft has a high antigenicity this causes an immune reaction against the donor bone by either a cellular or hormonal response which leads to the

rejection of the bone grafts. There are four categories for graft rejection [Urist (1980):

1. hyperacutic rejections - where there is an immediate antibody response which causes the deposition of fibrin, platelet aggregation, neutrophil aggregation and ultimate failure of incorporation;

2. acute rejection - where there is a delayed antibody response to cause the same results as observed in the hyperacute rejections;

3. chronic rejection - where there is a slow progressive antibody response to cause fibrous encapsulation of the graft;

4. intensified rejection - where there is an enhancement of the antibody response due to a previous graft which causes an intensification of an otherwise "normal" response.

Most rejected grafts will form an encapsulated fibrous tissue around the graft by a process called sequestration. This fibrous tissue prevents the grafted region from proceeding through the bone repair processes. The sequestration of a graft causes a blockage in the graft (biomaterial) incorporation stages via the inhibition of the cellular exchanges between the donor graft and recipient repair site, particularly the exchange of an osteogenic promotor (or growth factor).

The incorporation of the bone graft biomaterial has been outlined to progress in five stages similar to those processes for fracture healing, [see Figure 10, Urist (1980)]:

1. inflammation and proliferation of the preosteoblast and preosteoclast via the mesenchymal cells;

2. differentiation into osteoblasts;

3. osteoinduction;

4. osteoconduction;

5. restoration of the biomechanical and cellular function through remodelling.

These stages begin at the time the recipient receives the graft until years later, sometimes up to twenty years postoperatively. In the first stage, the "natural" biomaterial graft initially causes local inflammation. The mesenchymal cells are transported from the vascular system in the periosteum where they proliferate into preosteoblastic and preosteoclastic cells in the recipient bed. This has been postulated to chemically trigger BMP to aid in the migration of the cells towards the surface area of the bone graft. The response time for the proliferation of preosteoblasts and preosteoclasts in both autogeneic and allogeneic grafts are approximately the same, minutes to hours after the operation.

The second stage of differentiation of the preosteoblastic cells into osteoblast occurs within the first week postoperatively after the inflammation has diminished. New bone is deposited as the necrotic bone from the graft is slowly resorbed by osteoinduction and osteoconduction.

In the third stage of osteoinduction, the osteogenic growth factor(s) from the donor site transfer to the recipient site on a cellular level to induce the formation of new bone within the first four weeks. A cellular response to the osteogenic growth factor(s) is thought to be blocked by any delayed hypersensitivity reactions [Urist (1980)]. A delayed hypersensitivity reaction has been defined as the infiltration of perivascular tissues where a blockage of the capillaries in the zone of interdigitation that separates the donor bone from the recipient site, [see Figure 11]. The donor source (autogeneic or allogeneic) will determine this reaction time, for example homovital allografts have a shorter hypersensitivity time than homostatic allografts.

The fourth stage of osteoconduction involves the growth of new bone as it is conducted across the grafted area which begins within four weeks postoperatively. The osteoconduction stage requires the dynamic feedback between osteoinduction, the previous stage.

The fifth stage of restoration of the biomechanical and cellular functions includes the attainment of the mechanical stability, revascularization and ideally the restoration of the cosmetic appearance of the damaged area.

The rate of incorporation is determined by the amount of newly formed bone cells, the amount of inhibitory fibrous tissue and the age of the patient, for example the younger the patient the faster their remodelling process. The length of time required for each stage is dependent upon the recipient site (weight bearing versus non-weight bearing, recipient size (cavity versus large segment), local blood supply, and the degree of the immune response. The time is also effected by the surface area of the graft, donor source (cancellous versus cortical bone), donor type (autogeneic versus allogeneic versus synthetic), bone preparation, sterilization and storage techniques (chemical treatments, irradiation, shelf life and temperature).

Some of the advantages of using bone grafts are:

1. increase in the success of non-union repair;

2. increase in the osteogenic potential of the biomaterial by using homovital (live) autogeneic bone.

Some of the disadvantages of using bone graft are:

1. for autogeneic grafts, the patient must undergo an additional operation for *in situ* harvesting the graft;

2. patient discomfort, pain, instability, fracture fatigue, potential disfigurement, potential infections and damage of internal organs at the autogeneic donor site;

3. potential for surgical complications;

4. potential for donor-recipient mismatch that can lead to rejection of banked bone grafts or bone graft substitutes.

As early as the mid 1700's and mid 1800's, researchers have documented the osteogenic ability of excised bone [Prolo (1985)]. Some researchers have explored the applications of treated bone. Senn and co-workers in 1889 reported the experimental use of decalcified bone as a "delivery system for antiseptics in the treatment of bone cavities" [Mulliken (1980), Harakas (1984)]. The exploratory efforts of researchers such as Urist, Huggins and Reddi have revolutionized the knowledge and applications of non-autogeneic bone grafts (banked bone) as a biomaterial in the dental and orthopaedic field.

Prior to 1965 the applications of treated (banked) bone for the repair of large segmental defects had a low success rate. Urist attributes this to a variety of preparative treatments: the insufficient cleansing of soft tissues, irradiation sterilization, chemosterilization, storage temperatures and times. These preparative

treatments can decrease the efficancy of the osteogenic growth factor(s). Urist has identified the osteogenic factor as bone morphogenetic protein (BMP), which can be denatured during isolation procedures. It was not until 1965 that the inductive ability of decalcified (demineralized) bone was reported by Urist.

Through Urist's research efforts, a homostatic allogeneic bone was developed which would be sterile, have a lower antigenicity while preserving the activity of the bone morphogenetic protein. This homostatic allogeneic bone is referred to as autolyzed antigen-extracted allogeneic bone or AAA bone. The preparation of the AAA bone differs from the other types of homostatic bone graft materials, used experimentally, due to the treatment process. The AAA bone is prepared by treating the excised bone with:

1. 0.6N hydrogen chloride solution to demineralized the bone surface, to extract any acid soluble proteins, and to enhance the resorption of the graft by the recipient;

2. chloroform-methanol (1:1) solution to extract any lipids and lipoproteins;

3. 0.1M phosphate buffer solution at pH of 7.4 containing iodoacetic acid plus sulfhydral enzyme inhibitors and sodium azide to remove any soluble proteins that would enhance a delayed hypersensitivity reaction, to remove the osteocytes by autolytic digestion and to preserve the BMP.

These steps are followed by freeze-drying to ensuring the preservation of the BMP and other protein activity. Other researchers as Reddi and Huggins have used a particulate form of demineralized homostatic autogeneic or allogeneic bone.

b. Synthetic Graft Materials

Most of the current *in vitro* tissue culture models for testing new bone graft materials are based on the ability of undifferentiated mesenchymal cells from muscle (soft tissue) to differentiate into chondrocytes, a precursor of bone (hard tissue) [deJonge-Strobel (1987), Nathanson (1978), Takahashi (1986)]. This differientation is observed for osteoinductive materials. These *in vitro* experiments along with *in vivo* animal models can be used to determine the biocompatibility response of potential graft materials. Currently there is great interest in the development of bone osteoinductive biomaterials [summarized in Table 3]:

1. "natural" graft composites, for example allogeneic bone plus marrow from cancellous autograft;

2. implantable form of bone morphogenetic protein;

3. "synthetic" graft materials, for example calcium sulphate or calcium phosphate (in particular, hydroxyapatite) [Ricci (1986)];

4. composite mixture of a "natural" and "synthetic" materials, for example calcium sulphate and hydroxyapatite in conjunction with allogeneic, demineralized bone.

(1) Demineralized Bone Matrix & Bone Morphogenetic Protein

Demineralized bone matrix (DBM) is a "natural" biomaterial used in the repair of orthopaedic or dental defects. For clinical studies, the source is usually from allogeneic cortical or cancellous bone. For animal studies, the source is usually xenogeneic bone.

To obtain demineralized bone, first the excised bone must be cleaned of all soft tissue then treated with a dilute hydrochloric acid solution to remove the minerals, especially calcium. The procedure used to prepare the DBM is critical in maintaining the bioactivity of the osteogeneic growth factors within the material as reported by Urist [Iwata (1972)]. The resulting bone matrix contains collagen, residual proteins and polysaccharide with some cellular residues after the demineralization process. The bone can be demineralized on the bone surface only or throughout the entire bone depending on the length of decalification with dilute hydrochloric acid. The osteoinductive ability of this biomaterial has been demonstrated in both orthopaedic and dental applications using animal models or in clinical experiments.

This demineralization process not only removes calcium but also removes the bone derived growth factors which has been shown to be distinctively different from the osteoinductive factor [Canalis (1985), Hauschka (1986), Mohan (1984), Urist (1983)]. Bone stimulated growth by bone derived growth factor is reversible whereas bone stimulated growth by osteoinductive factor is irreversible. This proposed osteoinductive property of DBM has been attributed to a non-collagenous, diffusable protein called bone morphogenetic protein.

Experiments using DBM *in vitro* and *in vivo* have demonstrated that embryonic mesenchymal cells from muscle will differentiate into cartilage *in vitro* [Nathanson (1978, 1983), Nogami and Urist (1970), Urist (1970)] and further

mineralize to new bone *in vivo* [Aspenburg (1986), Bolander (1986), Gendler (1986), Glowacki (1981), Kaban (1982) Kohler (1987), Tuli (1981)]. This differientation is called chrondrogenesis. Animal studies have also demonstrated that *in vitro* DBM and BMP are not species specific but *in vivo* DBM is species specific and BMP is not species specific. Therefore xenogeneic DBM can undergo chrondrogenesis *in vitro* but not *in vivo*.

Bone morphogenetic protein (BMP) was first reported by Urist in 1965 as the osteoinductive factor isolated from demineralized bone. This isolated osteoinductive factor is an acidic polypeptide and glycoprotein that could bind to the hydroxyapatite naturally found in the inorganic phase of bone. Maintaining the activity of this osteoindictive protein has been postulated to be cruical in the diffusion of this protein out of the implanted DBM. The diffused protein then enters the defect site and targets the mesenchymal cells. This protein is thought to stimulate differentiation of the non-osseous mesenchymal cells into prechondrocytes, the precursor for cartilage cells. Cartilage cells will eventually lead to new bone cells.

Prior to 1965, researchers postulated that DBM had no effect as an osteoinductive factor. However, Urist observed that these findings were based on "improper" treatment of the DBM causing a denaturing of this critical protein.

The ability of DBM, with bioactive BMP, to provide an *in vivo* alternative for bone grafting has several advantages:

1. the relatively rapid osteoinduction of the material will stabilize the damage site prior to callus formation;
2. provides an unlimited source for grafting;
3. provides a source of the osteoinductive factor, the bone morphogeneic protein;

4. reduces the antigenicity common to most foreign materials;

5. eliminates the need for a painful operation required to excise the bone autograft;

6. avoids in situ time necessary to harvest autogeneic bone.

The clinical application of DBM, particularly BMP, as a viable orthopaedic biomaterial is still in the early stages of development. However, as additional studies are reported for the use of DBM/BMP, additional information will be revealed as to the inductive properties and clinical usefulness of this potentially important orthopaedic and dental biomaterial.

(2) Calcium Sulphate

Synthetically prepared calcium sulphate, $CaSO_4$, has been used for nearly a century in the repair of bone defects. The alpha hemi-hydrate of calcium sulphate, commonly known as Plaster of Paris, has been used during the last century to fill and repair bone defects.

Investigation of the implantation of synthetically prepared $CaSO_4$ by such researchers as Peltier have shown that $CaSO_4$ is biocompatible and bioresorbable. These characteristics are supported by the observations reported by Peltier:

1. does not stimulate a local tissue inflammatory response or systemic response;
2. does not inhibit normal bone growth processes in local or adjacent regions;
3. material is resorbed at a rate greater than the bone deposition;
4. does not change the Ca^{2+} concentration in the serum;
5. normal bone cells were found to occupy the site where $CaSO_4$ was resorbed.

The advantage of using $CaSO_4$ as a biomaterial for orthopaedic applications is that it "sets" well under physiological conditions, has good mechanical strength upon setting and is highly compatible with tissues. The disadvantages of using $CaSO_4$ are that it does not exhibit osteoconductive or osteoinductive abilities, its rate of resorption exceed the rate of bone deposition and the material will not set *in situ* if there is any blood or pus present in the implantation site. Therefore as a biomaterial, $CaSO_4$ has limited applications as an orthopaedic biomaterial unless used in combination with osteogeic, osteoconductive and/or osteoinductive materials, the rate of $CaSO_4$ resorption verus the rate of bone deposition can be

directed such that sufficient new bone is deposited at the implantation site to add stability during the repair precesses.

(3) Calcium Phosphate

Two crystalline chemical structures of calcium phosphate has been investigated for their biomaterial properties. The first has the apatite structure known as hydroyxyapatite (HA). Hydroxyapatite has a phosphate to calcium ratio of 1.67 as demonstrated in its chemical formula, $Ca_{10}(PO_4)_6(OH)_2$. The second has the beta whitlockite structure known as tricalcium phoshpate (TCP). Tricalcium phosphate has a phosphate to calcium ratio of 1.5 as demonstrated in its chemical formula, $Ca_3(PO_4)_2$. Both of these crystalline materials have been experimentally tested in their dense and porous ceramic form.

The density or porosity is controlled by the ceramic manufacturing processes. Dense calcium phosphates can be prepared by high isostatic pressure and sintering, hot-pressing, or precipitation-wet molding and sintering process. Porous calcium phosphates are usually prepared by adding a material to the calcium phosphate prior to the "moulding" followed by removal of this material by sublimation or decomposition to create voids or pores in the ceramic.

Researchers have observed similiarities and difference in the properties of these two ceramics as a biomaterials.

(a) Hydroxyapatite

The calcium phosphate naturally found in bone and teeth is hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. In bone, hydroxyapatite accounts for 60% of the inorganic (mineral) phase; and in teeth, it accounts for 98%. Hydroxyapatite has two functions in bone. The first is biomechanical by providing stability and rigidity to the hard tissue, bone. The second is metabolic by storing metabolic ions such as Ca^{2+}, Na^{+}, or Mg^{2+} and "waste" ions before detoxification.

Investigation has shown that naturally occuring hydroxyapatite and synthetically prepared hydroxyapatite are nearly identical in crystalline structure

though differ in porosity. Naturally found HA is highly porous while most synthetic forms are fairly dense with little to no porosity.

Various forms of synthetically prepared HA has been implanted in animals experimentally and in humans clinically [Frame (1987), Hoogendoorn (1984), Jarcho (1986), Krjei (1987), Moore (1987), Olivie (1987)]:

1. dense ceramic blocks
2. porous ceramic blocks
3. dense ceramic particulates with irregular shapes or uniform spheres.

The porosity of these ceramics has been defined in terms of the micro or macro porosity created during the synthetic processes. The macropores of the porous ceramic HA blocks is due to the "voids" created by the manufacturing process. The degree of macroporosity is controlled by chemical substance that is sublimed from the HA block. The micropores of the dense ceramic particulates is due to the channels formed between each dense HA particle of irregular or uniform shape. The degree of microporosity is controoled by the size and shape of the HA particles.

Research has demonstrated that HA is biocompatible, non-biodegradable, non-bioresorbable and osteoconductive. These characteristics are supported by the following observations of this material after implantation:

1. does not stimulate a local tissue inflammation response or systemic response;
2. does not interfer in the normal bone mineralization process;
3. integrates well with homovital bone without fibrous encapsulation of the implant;

4. provides a medium for new bone to be deposited by directing the new growth through the material's pores;

5. demonstrated the ability to retain bone density in areas that may have otherwise loss bone density through resorption.

Since porous ceramic blocks and dense particulate HA have channels for the deposition of new bone, many researchers have investigated these two forms of HA as medical alternatives for the repair of bony defects in both dental and orthopaedic applications.

Dense particulates of HA have several advantages over porous blocks in that the particulate HA will more readily pack into any shaped implantation site, the material does not have to be pre-shaped by the surgeon to fit the implantation site and the degree of porosity can be controlled by using different sizes or shapes of HA. One disadvantage of particulate HA is that there is a tendency for some of the particles to dislodge and migrate from the implantation site before they can be anchored in place by new bone. Another disadvantage is the difficulty in controlling and maintain the tight packing of the particules. This tight packing is critical in directing the osteoconduction of new bone between the micropores of the pores.

Therefore as a biomaterial, the particulate form of HA has limited applications as an orthopaedic biomaterial unless the migration of the particles and the size of the micropores between the particles can be directed and controlled. These advantages and disadvantages lead to the investigation of combining HA with other materials. For example, the use of particulate HA in combination with $CaSO_4$ [Ricci (1986)].

(b) Tricalcium Phosphate

The other type of calcium phosphate which has been investigated has the beta whitelockite structure. Tricalcium phosphate, $Ca_3(PO_4)_2$, possesses most of the same characteristics as hydroxyapatite (the apatite structure). These similiarities in the biomaterial characteristics have been attributed to their similiar chemical structures, particularly their close Ca/P ratios.

Research has demonstrated that TCP is biocompatible, partially bio-resorbable and osteoconductive (though slightly less than HA). The partial bio-resorbability of TCP has been linked to the size of the micropores produced in the manufacturing process. The bio-compatibility and osteoinduction were supported by observations made by such researchers as Bowers (1986), Froum (1987) and Jarcho (1986).

c. Synthetic Graft Composite of Hydroxyapatite with Osteogenic Factor

Researchers have vigorously explored the possibilities of developing a clinically useful "natural" and/or "synthetic" biomaterial for use as a bone graft substitute in the last two decades. Research in this area has established [see Table 3]:

1. homovital autogeneic bone is biocompatible and osteogenic;

2. homostatic autogeneic or allogeneic (banked) bone is biocompatible and osteoconductive or osteoinductive depending upon whether the activity of the BMP is maintained;

3. hydroxyapatite, a natural mineral of bone, in the synthetic form is biocompatible, slowly biodegradable and osteoconductive;

4. whole, sliced or particulate demineralized bone matrix is biocompatible and osteoinductive;

5. bone morphogenetic protein is biocompatible and highly osteoinductive.

The ideal bone graft material should have the following properties to ensure close to 100% restoration of the mechanical and cellular functions:

1. reduced antigenic response;

2. stimulate osteogenesis, osteoconduction or osteoinduction;

3. non-toxic, non-carcinogenic and non-immunogenic to surrounding tissues;

4. controlled osseous growth;

5. sterilizable;

6. good shelf life;

7. good handling properties for implantation by the surgeon;

8. cosmetically appealing bone repair with minimal deformity of the bone due to the graft;

9. maintenance of the mechanical integrity of the bone especially for those bone segments which bear weight.

A composite material of true osteoinductive properties may be clinically possible by incorporating the known *in vitro* and *in vivo* properties of demineralized bone matrix, bone morphogenetic protein, calcium sulphate and hydroxyapatite.

Currently researchers are investigating the extent of bone incorporation of a composite biomaterial that consists of a mixture of a partially resorbable, osteoconductive and osteoinductive material to improve the osteogenic response of a grafting material for bone repair. Urist et al has reported on the osteoinductive properties of demineralized bone matrix and bone morphogenetic protein [Syftestad (1979), Urist (1983)]. Parsons et al has reported on a "partially resorbable" osteoconductive composite material consisting of calcium phosphate and particulate hydroxyapatite [Alexander (1987), (Ricci (1986)]. It would be of interest to investigate the extent of bone incorporation of a composite mixture of a partially resorbable, and an osteoinductive material.

The research in the following sections describes the *in vitro* investigation of the biocompatibility and inductive effects of the major biological components for a "synthetic" bone graft material. The materials investigated were demineralized bone matrix (DBM), devitalized bone matrix (DVBM) and osteoinductive factor, bone morphogenic protein (which has been added to devitalized bone matrix). Specifically these *in vitro* experiments are designed to demonstrate the inductive activity of the osteoinductive protein when absorbed on non-active DVBM. Results

are to be compared to DBM (positive control - active indicator) and DVBM (negative control - inactive indicator).

METHODS

The powdered demineralized bone matrix was prepared in-house by decalifying cleaned cortical bone from rabbit tibiae and femurs with dilute hydrochloric acid, ethylene diamine acetic acid, lithium chloride and calcium chloride according to the Method II procedure described in Appendix 5. Essex Medical Products, Inc. (Wheat Ridge, CO) supplied the laboratory with additional rabbit demineralized bone matrix (according to Method III) along with rabbit devitalized bone matrix and rabbit devitalized bone matrix with bovine bone morphogenic protein [see Appendix 5]. These materials were prepared according to the Method III procedures described in Appendix 5.

All glassware, culture dish rings and wire mesh shelves were autoclaved in high pressure and temperature for sterilzation. All cell culture solutions (CMRL-1066 and Hank's Balanced Salt Solution) were prepared within 24 hours of each experiment [see Appendix 4]. The pregnant Sprague-Dawley rats were timed such that the fetuses were twenty days old at the time of the experiment.

Several hours prior to the experiment, the laminar flow or UV hood surface was sterilized with 70% ethanol and the cleaned surgical tools were soaked in a 70% ethanol bath for one hour with the air flow or UV lamp turned on. The sterile welled culture dishes were assembled [see Appendix 5] and the vitrogen was prepared [see Appendix 4].

Embryonic (fetal) muscle was excised from the hind thighs of twenty day fetal Sprague-Dawley rats and placed in CMRL-1066 culture medium under sterile conditions. All skin, cartilage, bone, connective tissue, blood vessels and non-muscular tissue was removed. The "cleaned" muscle was teased into small bundles and temporarily stored in the culture medium on ice.

The lypholized bone matrices were removed from the freezer. The amount needed for each experiment was transferred to a sterile petri dish under a laminar flow or UV treated hood. The bone matrix materials were rehydrated with 0.5-1.0 milliliters of sterile chicken plasma for 15-20 minutes.

The *in vitro* tissue culture model described by Nathanson (1978) was modified since a powdered form of the demineralized bone matrix, devitalized bone matrix and devitalized bone matrix with bone morphogenetic protein were used. The modifications to this tissue culture model entailed the use of liquid collagen, tradename vitrogen. The vitrogen was used to hold the particles of the bone matrix together in culture. A bed of partially gelatinized vitrogen was placed on top of the wire mesh shelf which rested on the rim of the inner well of a welled culture dish. The wire mesh shelf held the cell cultures, the inner well held the culture medium which provided "nutrients" to the cultures and the outer well held distilled deionized water which maintained the humidity inside the culture dish.

The rehydrated bone matrices and fetal rat muscle were combined together on the vitrogen bed such that the muscle was layered between the bone matrix. The cell cultures were maintained for 16-20 days by: (1) changing 50-75% of the volume of the culture medium every two to three days and (2) adding additional water to the outer well to ensure that the himidity inside the culture dishes were maintained.

The second modification to the model described by Nathanson (1978) was the procedure used to infiltrate and embed the cell cultures. The cell cultures containing powdered bone bone matrix could not be sectioned when embedded in paraffin. Paraffin embedding was used for the sliced demineralized bone used by Nathanson. The particles of the bone matrix separated from the paraffin embedded cultures due to the differences in the hardness of the bone matrix verus the softness of the paraffin.

The cell cultures were fixed in 10% neutral buffered formalin, dehydrated with ethanol, cleared in xylene, infiltrated with methylmethacrylate and embedded in poly methylmethacrylate [see Appendix 5]. The embedded samples were sectioned in 5 micrometer slices on a Riechert-Jung Polycut E Microtome, mounted on coated glass slides, stained and examined under light microscopy for the presence of cartilage formation. After initial sectioning, the embedded samples were cut into three to four sections on a diamond saw, re-embedded in poly methyl methacrylate with the proper orientation and carefully labelled. Each embedded region, labelled A through D, was sectioned in 5-6 micrometer slices on the microtome, mounted, stained and examined under light microscopy for the presence of cartilage formation.

RESULTS

The plastic embedded cell cultures consisting of powdered demineralized bone matrix, prepared according to Method II [see Appendix 5], showed the formation of cartilage under light microscopy [see Figure 13]. The cartilagenous matrix contained proteoglycan as demonstrated by the pink staining matrix from Periodic Acid Schiff(PAS)-Alcian Blue stain and the light green staining matrix from Goromi's Trichrome stain. The production of proteoglycan is an indicator of the formation of cartilage and the presence of chondrogenesis.

The plastic embedded cell cultures consisting of devitalized bone matrix, prepared according of Method III [see Appendix 5], showed a necrosis of the muscle which was surrounded by this devitalized bone matrix. Under light microscopy, there was no evidence that the mesenchymal cells differentiated into cartilage or that there was the production of proteoglycan [see Figure 14].

In cell culture, the devitalized bone matrix containing the addition of the osteoinductive factor also showed a necrosis of the muscle with no chondrogenesis of the mesenchymal cells [see Figure 15]. The source of the osteoinductive factor was bovine bone morphogenetic protein prepared according to the procedure described in Appendix 5.

DISCUSSION

The differentiation of mesenchymal cells to cartilage has been demonstrated *in vitro* by Nathanson (1978) in a fetal (embryonic) muscle cell culture model. This model showed the chrondrogenesis of fetal muscle when grown on sliced demineralized bone. Their research has shown that the demineralization process is one crucial factor in maintaining the vitality of the muscle cells in culture.

In this modified fetal muscle cell culture model, fetal rat muscle was grown on powdered demineralized bone matrix, devitalized bone matrix and devitalized bone matrix with osteoinductive factor. Liquid collagen, vitrogen, was used to hold the bottom and top layers of the bone matrices together in culture. The vitrogen did not appear to infer with the differientation of the mesenchymal cells as demonstrated in formation of cartilage from the cell cultures containing demineralized bone matrix, see Figure 13.

The positive response of fetal muscle grown on particulate demineralized bone matrix was consistant with the research of Nathanson (1978, 1985), Harakas (1984) and Landesman (1986). The necrosis of the fetal muscle grown on particulate devitalized bone matrix supports the premise that this devitalized bone no longer has its osteoinductive properties. However, the presence of necrotic muscle from muscle grown on devitalized bone matrix with the addition of the osteoinductive factor was not expected.

The observed absence of cell proliferation and cartilage formation from the cultures containing devitalized bone matrix (DVBM) and devitalized bone matrix with osteoinductive factor (DVBM+BMP) raises two questions. The first is whether these observations are due to the preparation of the devitalized bone matrix (Method III) or the bone morphogenetic protein [see Appendix 5]. The second is whether these observations are due to the experimental conditions used

for the cell culture experiments. To arrive at a plausible conclusion, one should examine the following possible factors:

1. the osteoinductive factor, bovine bone morphogenetic protein, may have been inactive (no osteoinductive activity);

2. there was a flaw in the cell culture technique which may have caused the necrosis of the fetal muscle. Therefore the necrosis observed for both DVBM and DVBM+BMP may have been due to the experimental techniques and not indicative of the bone matrix materials.

3. the method III for preparing the bone matrices could cause necrosis of the fetal muscle *in vitro*. For example, any residual acid remaining in the bone matrix after the demineralization procedure can destroy the mesenchymal cells in culture.

4. this modified *in vitro* cell culture model may not be the ideal conditions for demonstrating the osteoinductive activity of the bone morphogenetic protein.

The probable cause of the observed muscle necrosis for the cultures with DVBM+BMP is more than likely not due to the inactivity of the BMP. *In vivo* experiments with the identical material by Damien (1989) demonstrated the osteoinductive activity of the BMP. These *in vivo* experiments showed the formation of ossicles in the muscle pouches of rabbits after implantation of particulate DVBM with BMP.

To rule out the second possibility of a flaw in the cell culture experiment, additional experiments would have to be conducted. Assuming that necrosis of the fetal muscle was still observed, one could conclude that the necrosis is due to either

the procedure used in processing the material or due to the modified cell culture model.

According to an earlier consultation with Nathanson, the processing of the demineralized bone for cell culture experiments is important. For example, in the demineralization process used in Method II, the bone was also treated with EDTA, LiCl and $CaCl_2$ verses Method I and III. The presence of small amounts of acid can acidify the culture medium and/or destroy the muscle cells.

The modifications to the cell culture experiments, specifically the use of the powdered bone matrices, the osteoindictive factor and the vitrogen, may not have been sufficent to demonstrate the differentiation of the mesenchymal cells grown on the bone matrix with the osteoinductive factor. Other modifications may be necessary to show the chrondrogenesis of muscle grown on devitalized bone matrix with the addition of the osteoinductive factor.

CONCLUSION

1. The demineralized bone matrix used in this research exhibited the properties of an osteoinductive material *in vitro* by the formation of cartilage. This formation of cartilage resulted from the differentiation of the mesenchymal cells which were derived from the fetal rat muscle.

2. The devitalized bone matrix used in this research exhibits no effect on the chondrogenesis of the mesenchymal cells. In fact these cell were shown to be necrotic. Therefore, one can conclude that this material does not contain the viable proteins or factors necessary for osteoinduction or osteoconduction to take place.

3. The devitalized bone matrix with the addition of the osteoinductive factor, bone morphogenetic protein, exhibited no effect on the chondrogenesis of the mesenchymal cell.

The necrosis of the mesenchymal cells for devitalized bone matrix and devitalized bone matrix with bone morphogenetic protein is probably due to insufficent removal of the acid after demineralization or due the methodology used for the *in vitro* experiments. Further research would be necessary to establish the probable cause of these findings, particularly for the unexpected necrosis of the muscle grown on devitalized bone matrix with the addition of bone morphogenetic protein.

APPENDIX 1

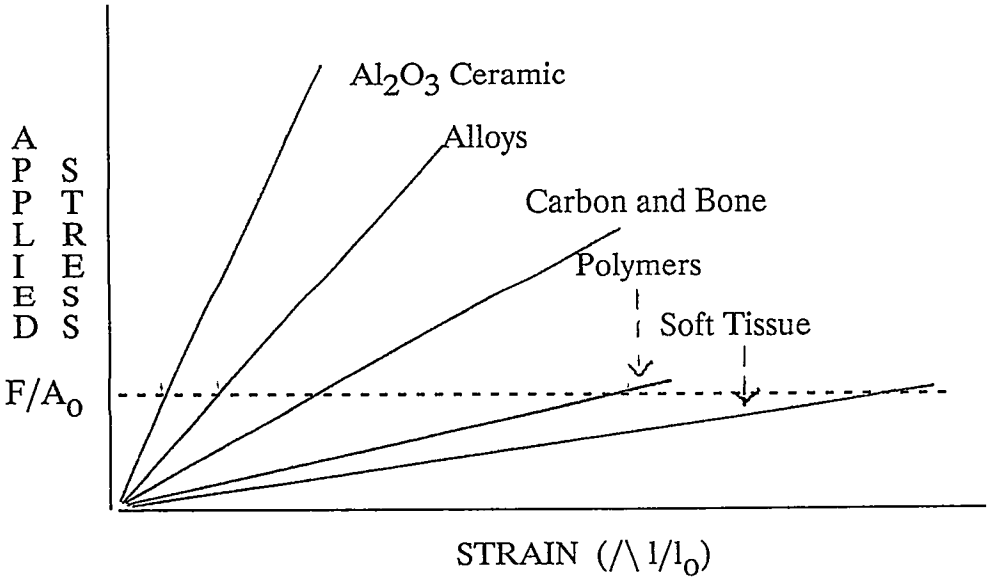


Figure 1 - Stress versus Strain Curve for Various Biomaterials [Lemmons (1986)]

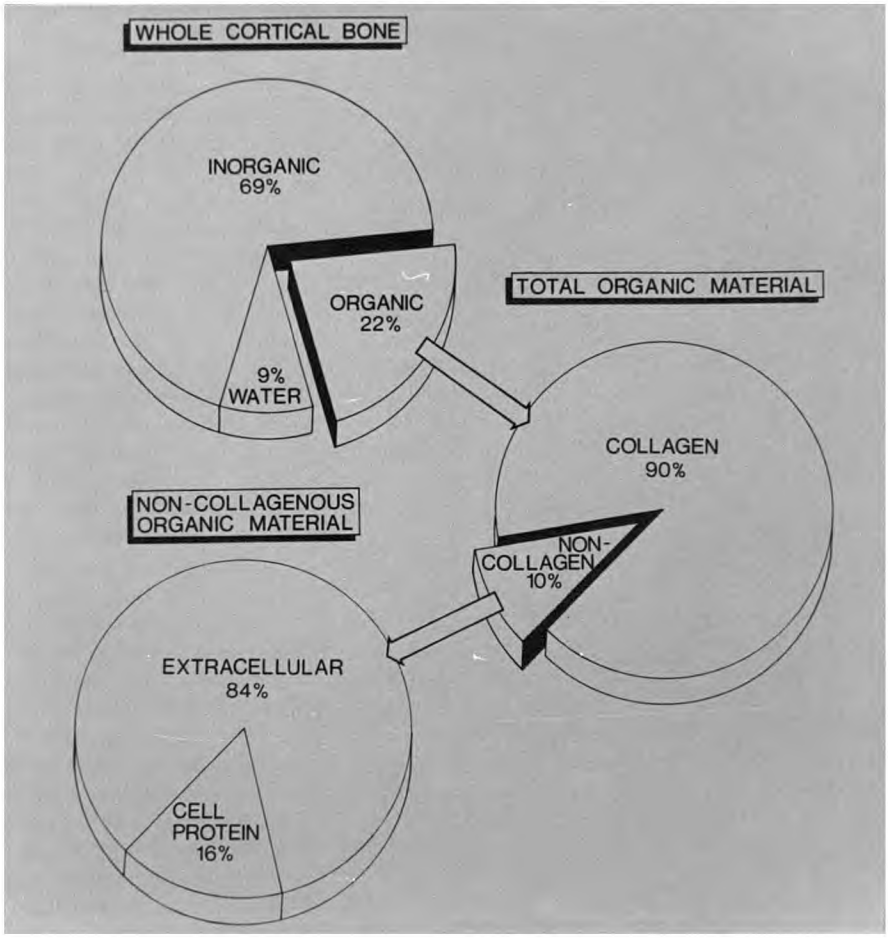


Figure 2 - Structural Percent Composition of Cortical Bone [Matthews (1980)]

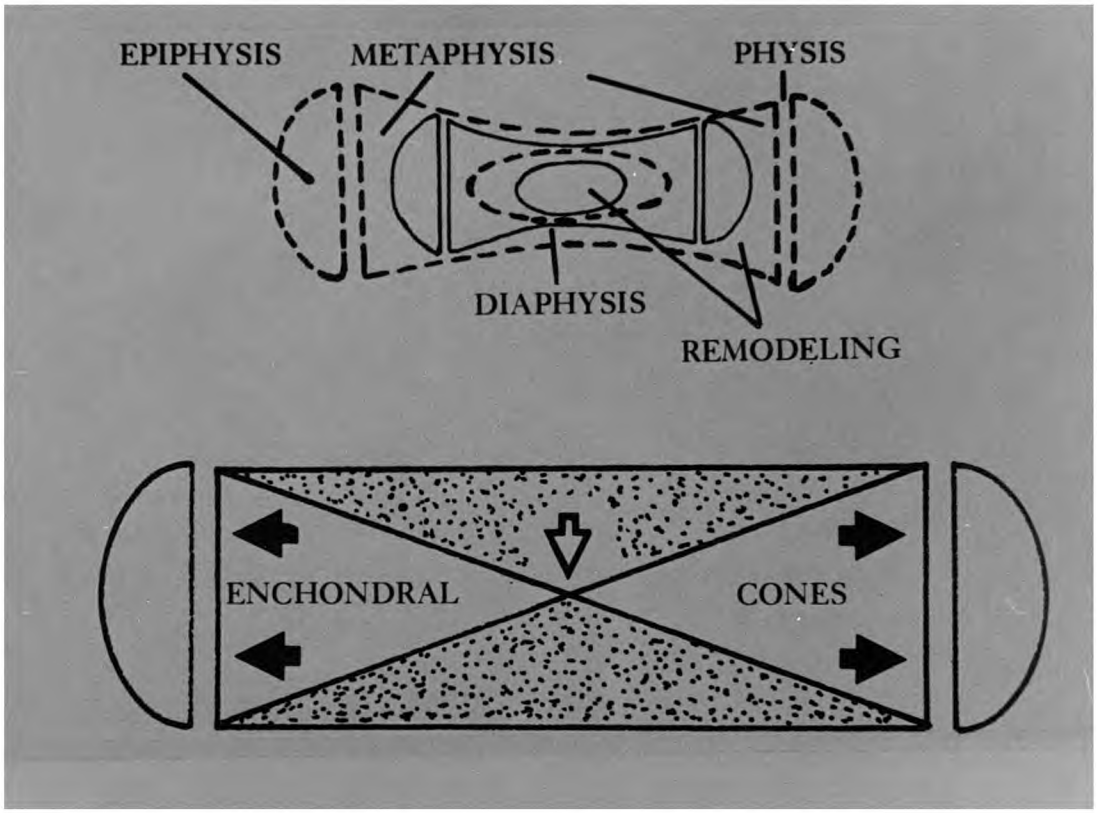


Figure 3 (a) - Schematic of the Regions for Radial Growth towards the Endochondral Growth Plate [Ogden (1980)]

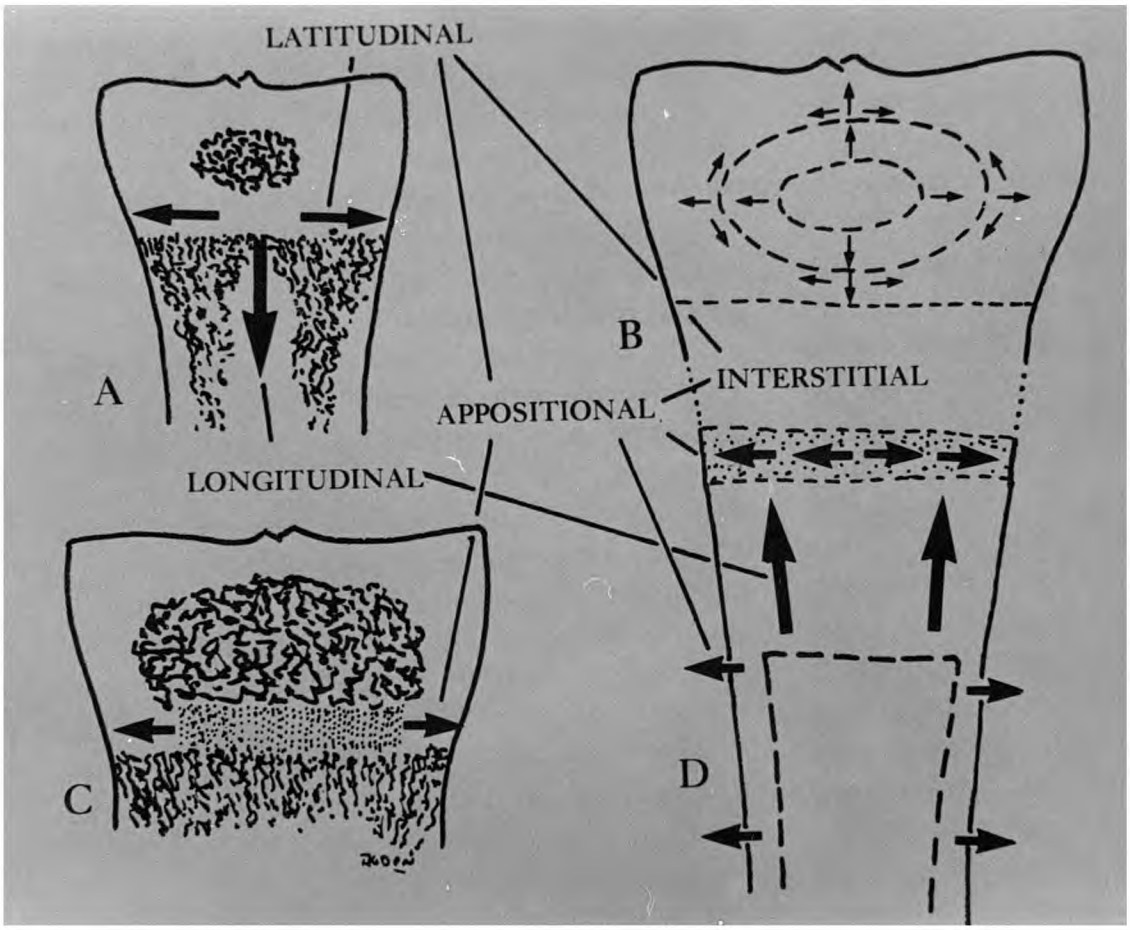


Figure 3 (b) - Schematic of the Growth Patterns towards Endochondral Growth Plate [Ogden (1980)]

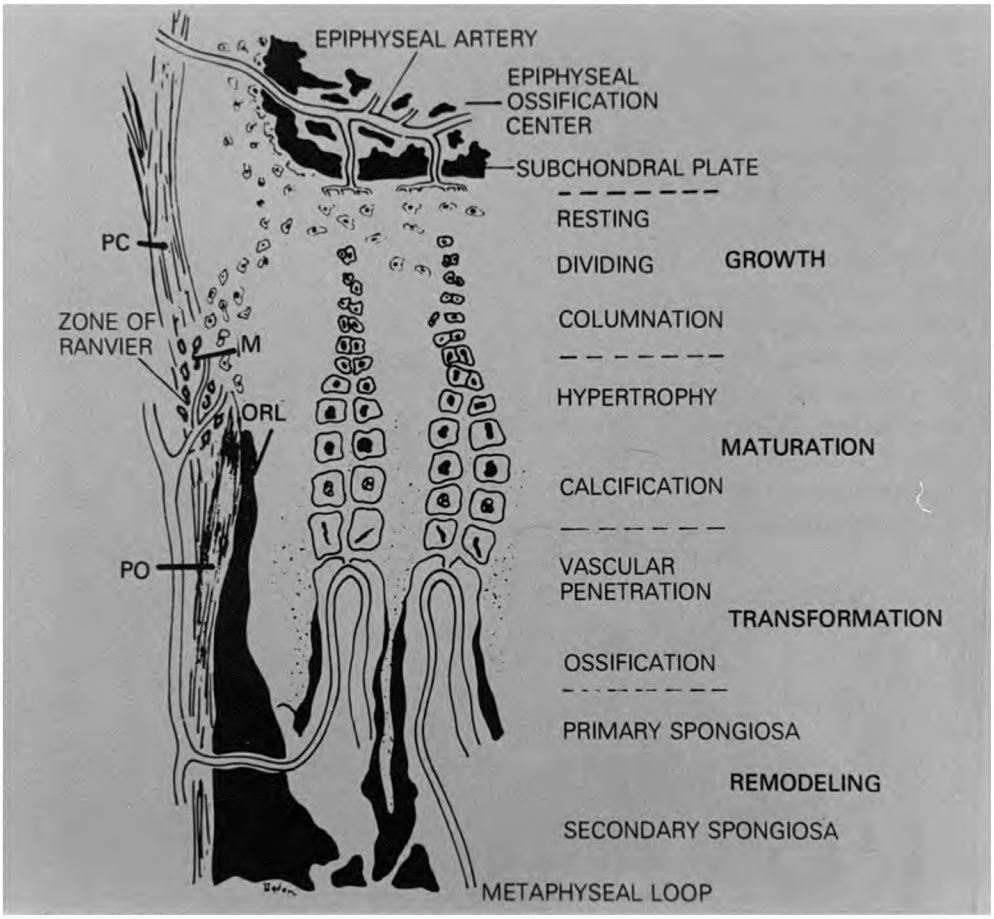


Figure 4 (a) - Schematic of the Zones at Endochondral Growth Plate [Ogden (1980)]

Figure 4 (b) - Lateral Cross Section of Femoral Cartilage at Endochondral Growth Plate [Ogden (1980)]
a-proliferation zone, b-hypertrophy zone, c-provisional zone

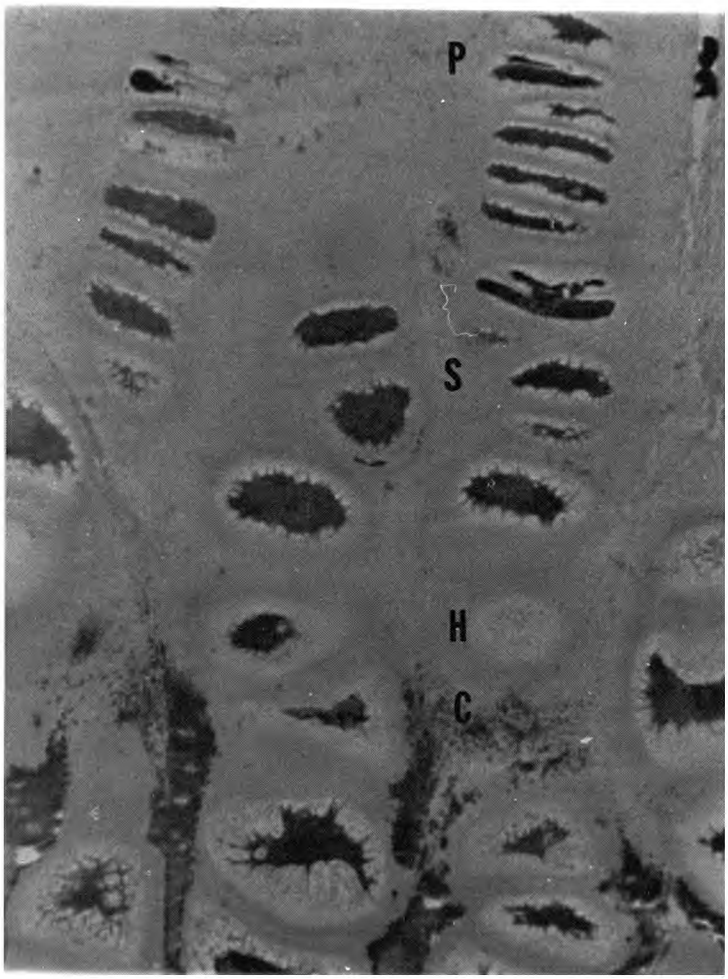


Figure 4 (c) - Photomicrograph of Endochondral Growth Plate
(x600) [Ogden (1980)]
P-proliferation zone, S-matrix synthesis zone,
H-cell hypertrophy zone, C-provisional calcification zone

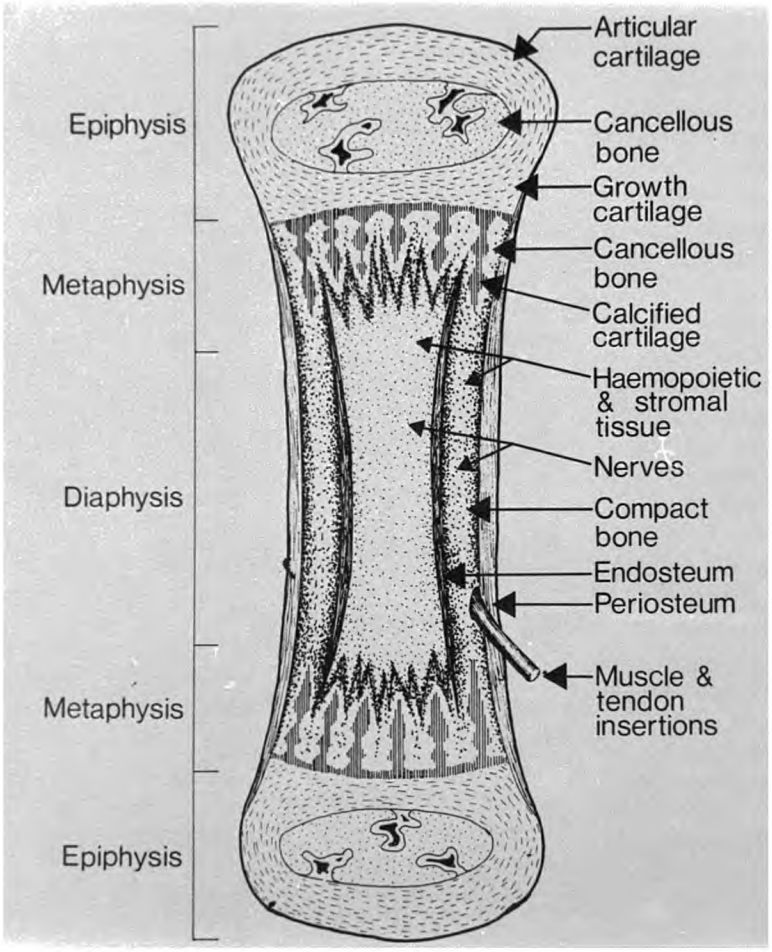


Figure 5 - Sketch of Bone with Tissue
[Triffitt (1980)]

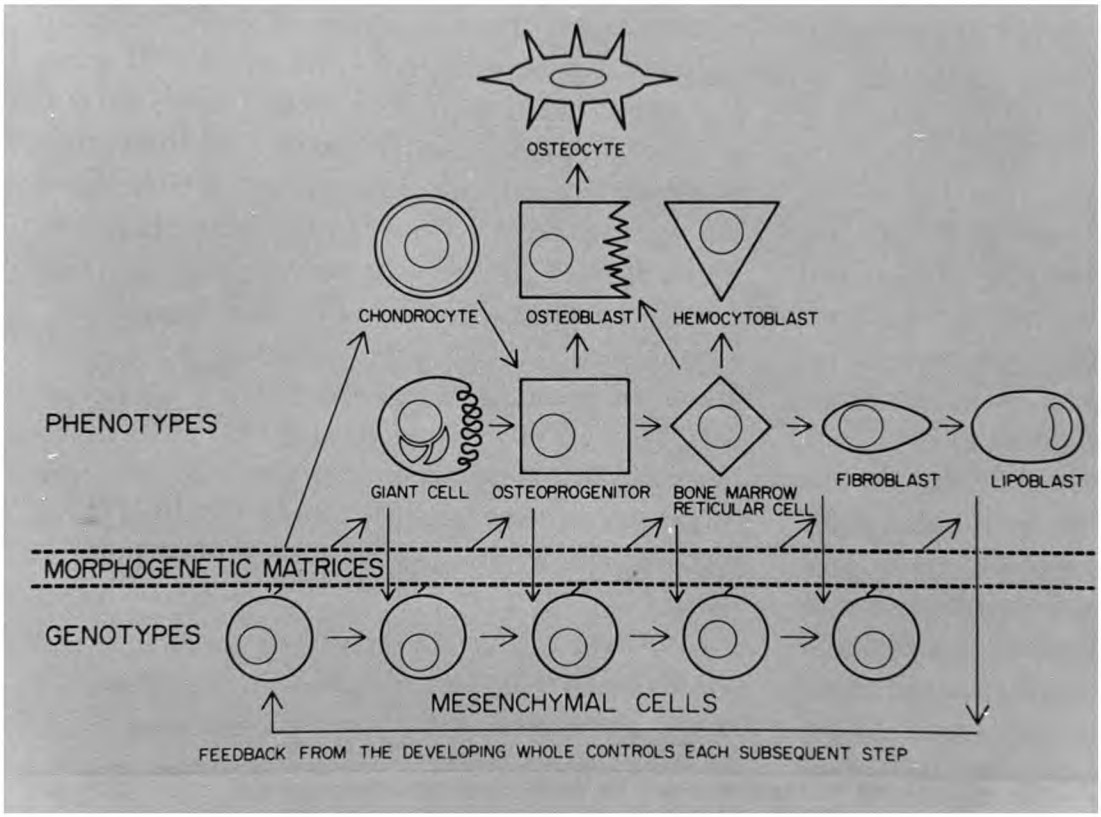


Figure 6 - Six Cell Types of Bone

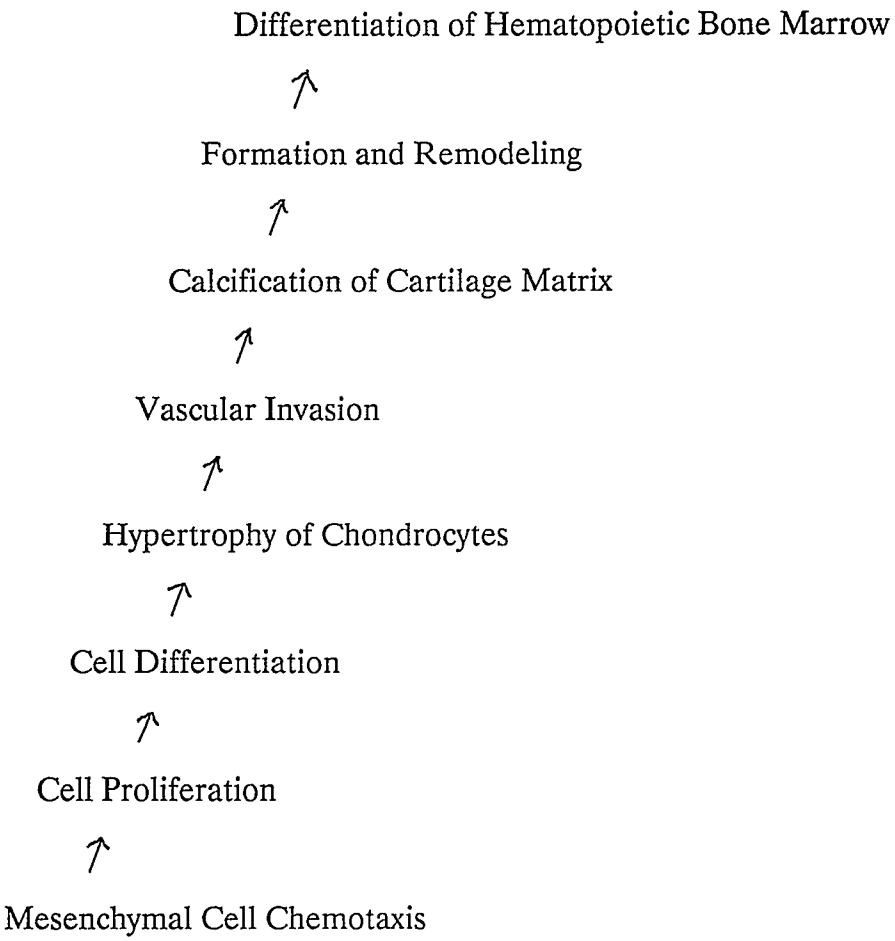
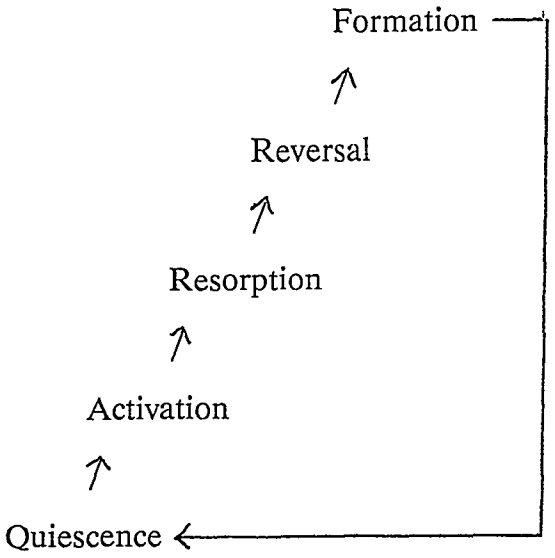


Figure 7 - Endochondral Bone Differientation Phase Diagram
[Sampath (1981)]

(a)



(b)

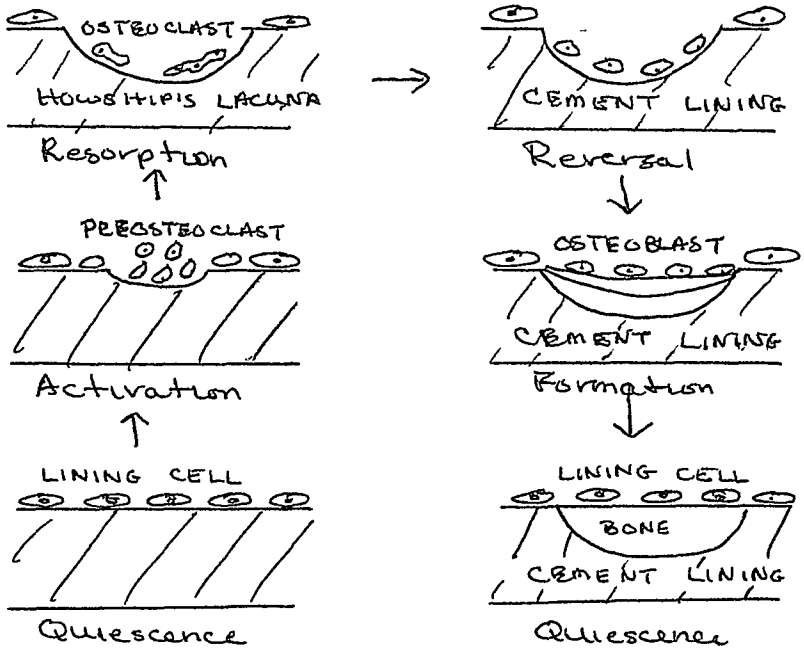


Figure 8 (a) - Cyclical Remodeling Phase Diagram
8 (b) - Parfitt's Five Stage Cellular Model for Cyclical Remodeling [Parfitt (1984)]

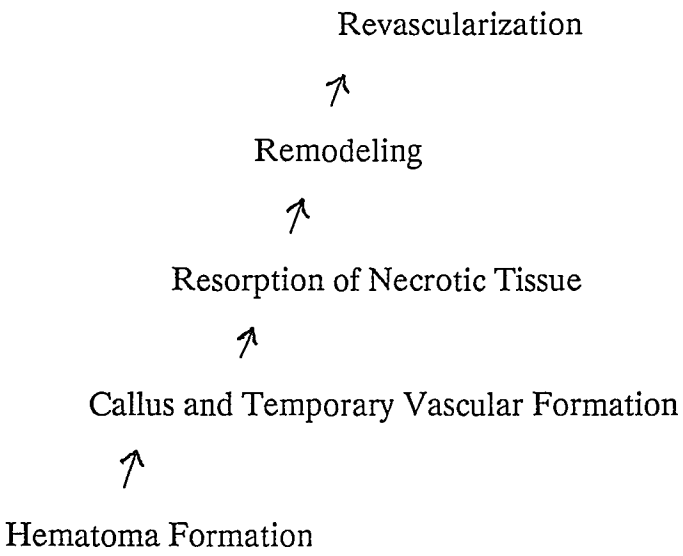


Figure 9 - Fracture Healing Phase Diagram
[Simmons (1985)]

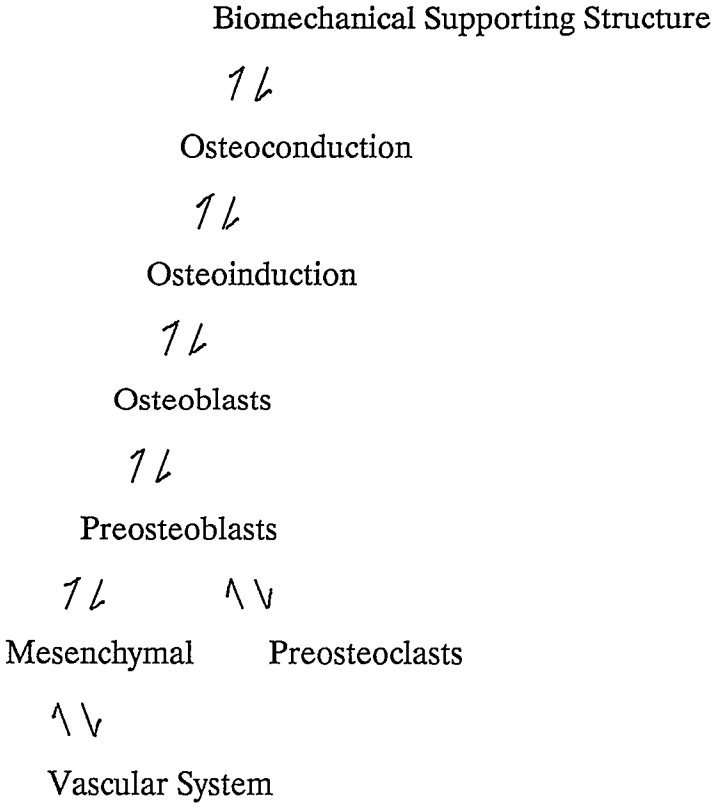


Figure 10 - Bone Graft Incorporation Phase Diagram [Urist (1980)]

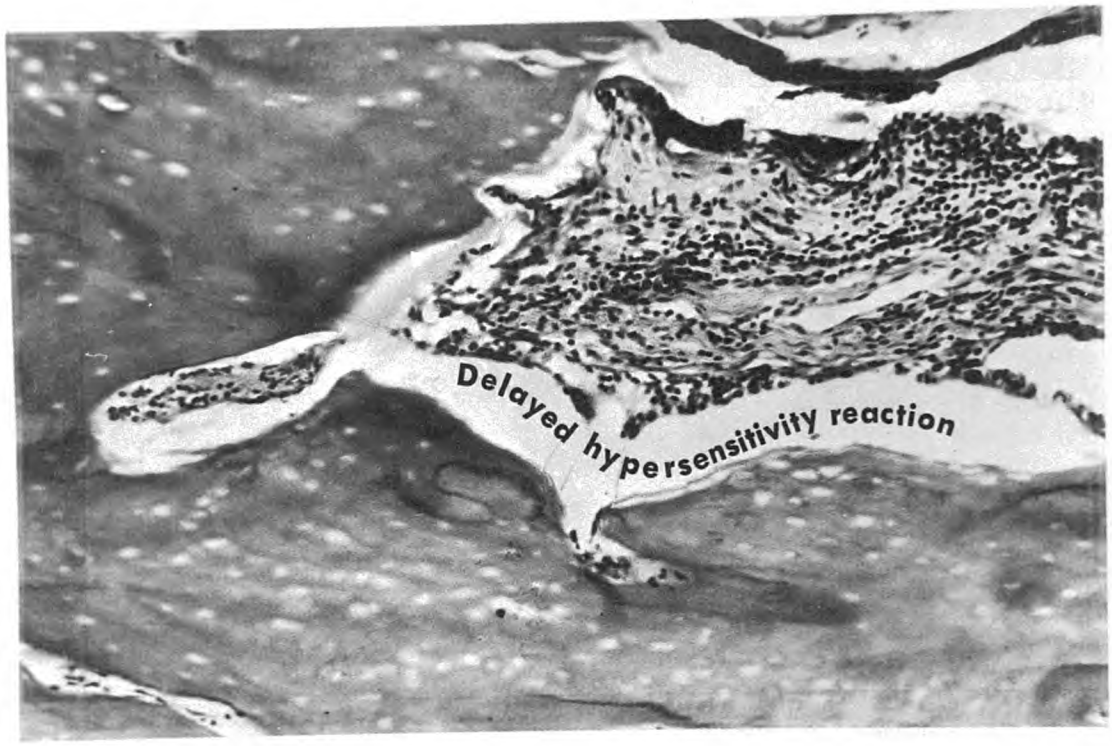


Figure 11 - Site of Delayed Hypersensitivity Reaction at the Zone of Interdigitation [Urist (1980)]

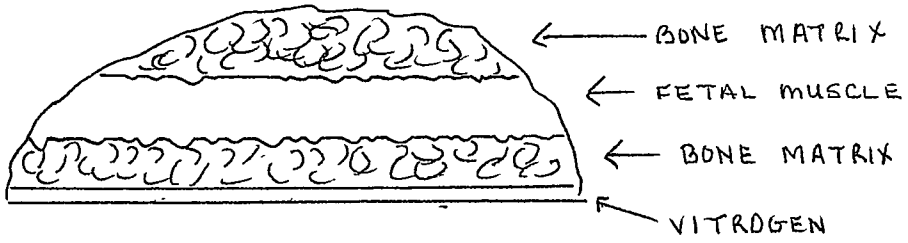


Figure 12 - Sketch of Cell Culture Layers

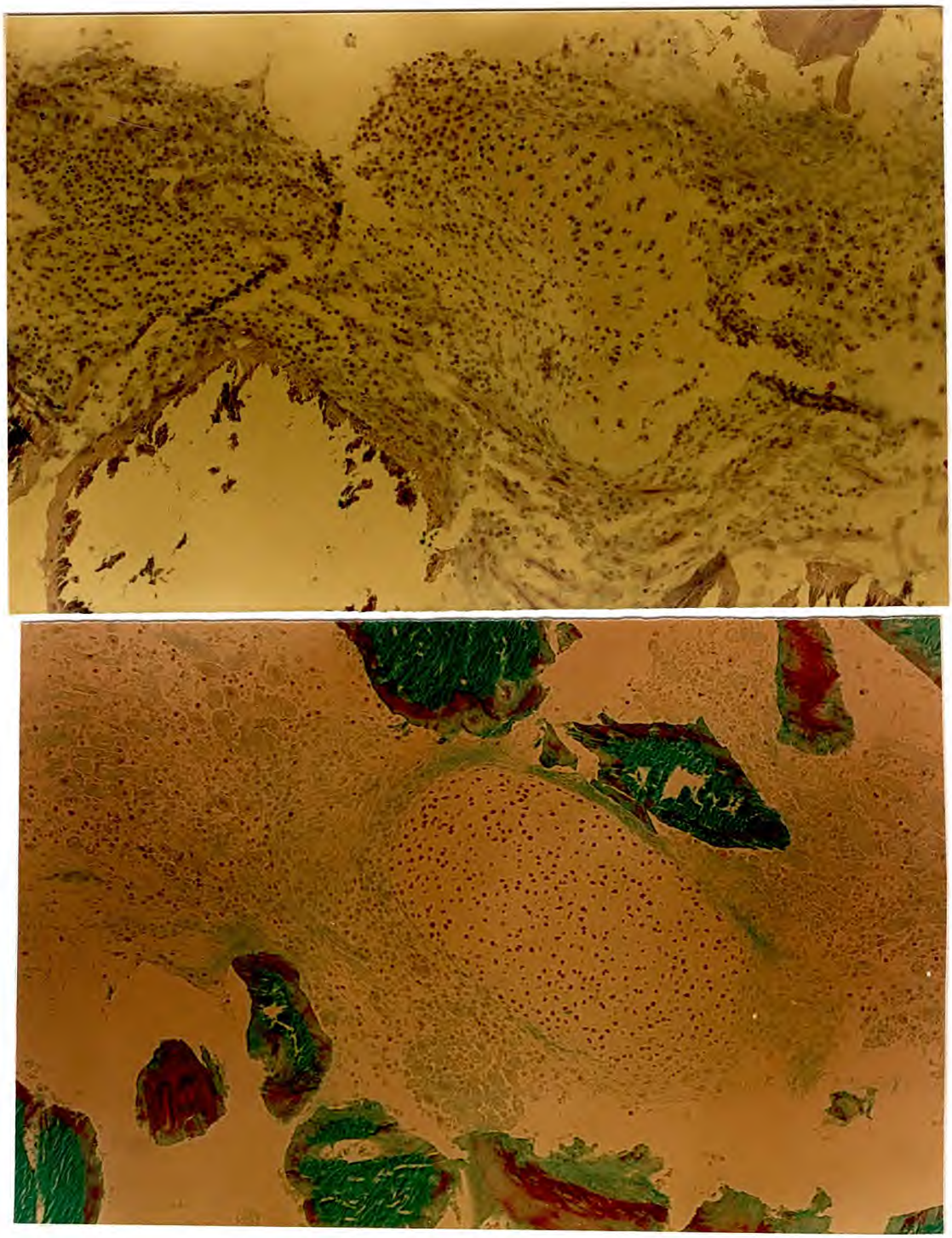


Figure 13 - Mircrographs of Cell Culture with Powdered Demineralized Bone Matirx (x62.5)
top - PAS stain bottom - Trichrome stain

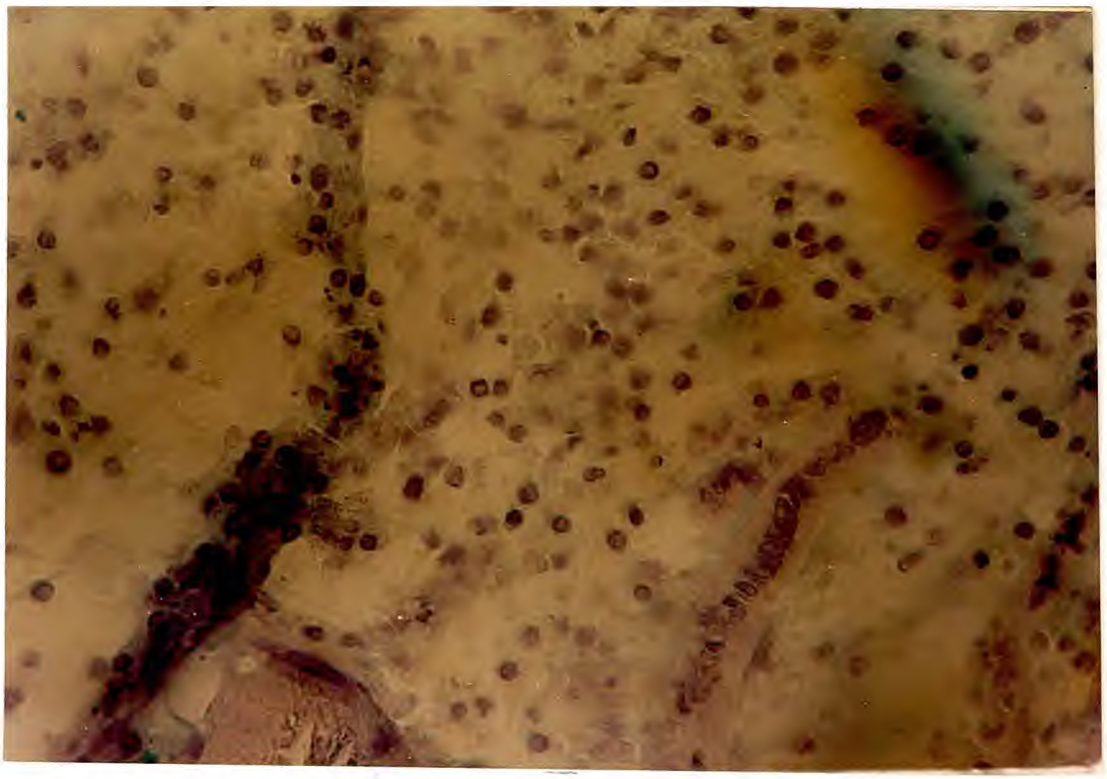


Figure 13 (continued) - Mircrographs of Cell Culture with Powdered Demineralized Bone Matirx (x125)
PAS stain

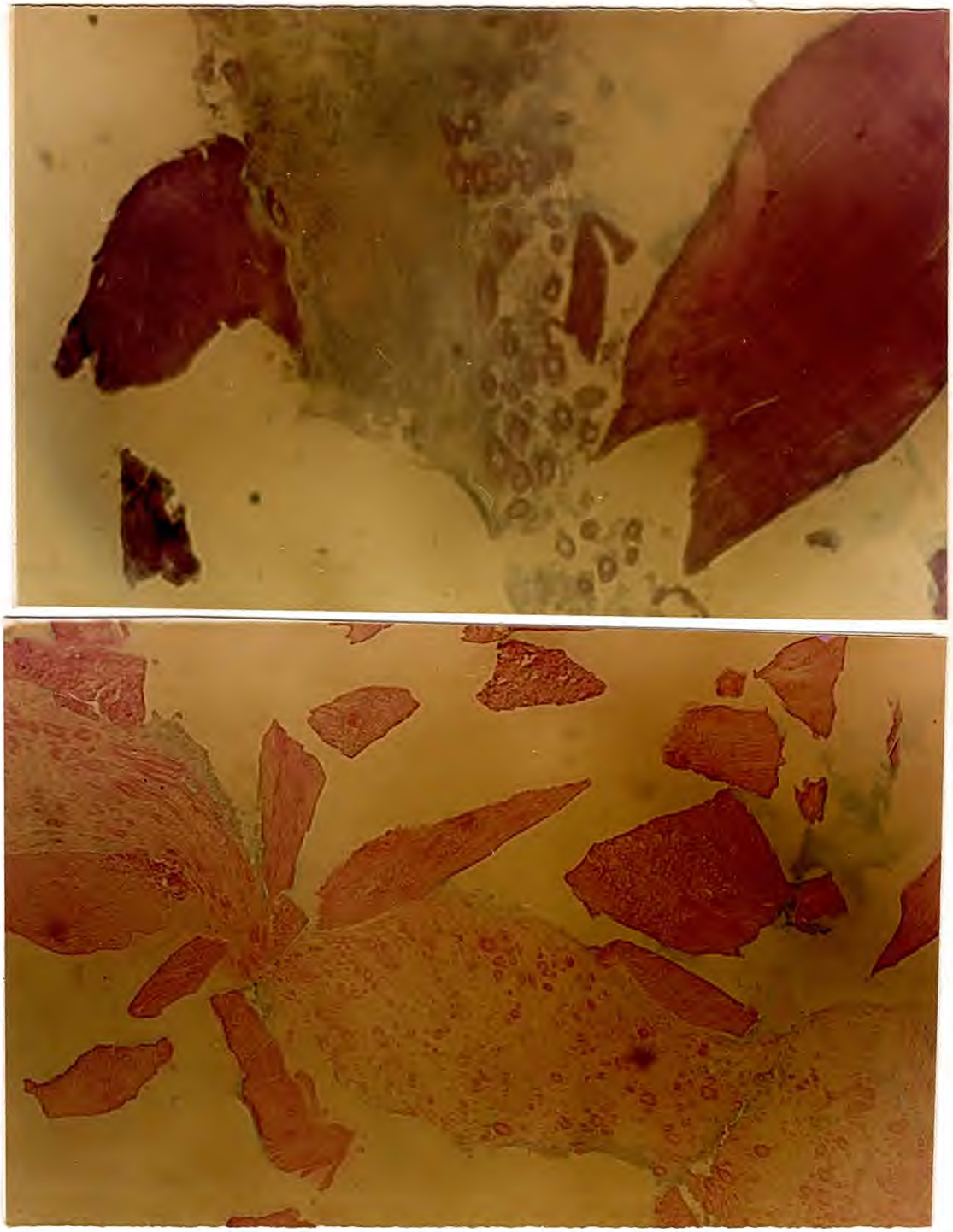


Figure 14 - Micrographs of Cell Cultures with Devitalized Bone Matrix (x62.5 top, x31.25)
PAS stain

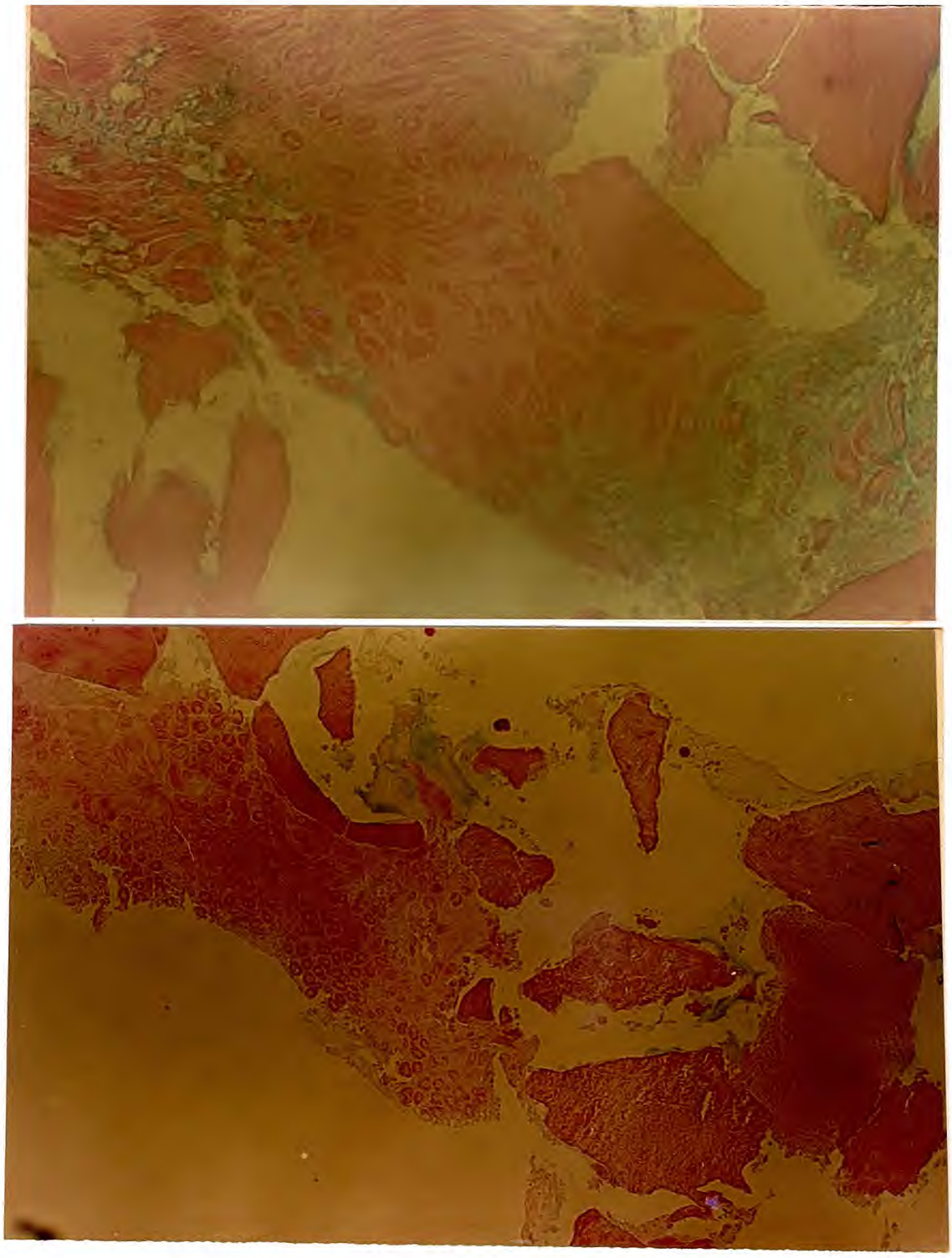


Figure 15 - Micrographs of Cell Cultures with Devitalized Bone Matrix and Osteoinductive Factor (BMP)
(x62.5 top, x31.25) PAS stain

APPENDIX 2

Material	Young's Modulus (GN/m^2)	UTS (MN/m^2)	Elongation (%)
Alumina	350	*	<1
Stainless Steel	220	1000	10
Co-Cr-Mo	220	700	5
Bone	**17**	**130**	**2**
PMMA	3.0	70	5
Nylon 66	2.8	85	100
UHMW Polyethylene	0.5	50	500

* value is extremely varible, but polycrystalline material is brittle when tested in tension.

TABLE 1 - Mechanical Properties of Bone Compared to Various Engineering Materials

Specimen	Young's Modulus (GN/m^2)	Poissons Ratio/UTS ($/MN/m^2$)	Compressive Strength (MN/m^2	Elongation (%)
Human Long Bones				
//long axis	17.0	0.46 / 132	192	3.4
⊥ long axis	11.5	0.58 / 61	130	
Human Femoral Wet Bone (in tension)				
femur:				
40-49yrs	17.7	/ 140	209	3.4
60-69yrs	17.1	/ 129	179	2.5
tibia:				
40-49yrs	28.8	/ 170	204	2.9
60-69yrs	19.9	/ 147	183	2.7
Human Cortical Bone				
31 years	20.0		158	0.92
61 years	19.0		120	0.65
Embalmed Human Cortical Bone				
femur:				
41 years	14.6	/ 100		1.3
71 years	13.5	/ 67		1.1
tibia:				
41 years	18.5	/103		1.7
71 years	15.6	/ 83		1.6

TABLE 2 - Mechanical Properties of Bone

	BC	BR	OC	OI	OG
Hydroxyapatite					
dense block	X	O			
porous block	X	O	X		
dense particulate	X	/	X		
Tricalcium Phosphate	X	/	X		
Calcium Sulphate	X	X			
Bone					
autogeneic graft	X	O			X
banked					
allogeneic or autogeneic	X	O	X	X	
demineralized bone marix	X	O		X	
bone morphogenetic protein	X			X	

Key: BC = biocompatible BR = bioresorbable

OC = osteoconductive OI = osteoinductive OG = osteogeneic

O = none / = partially X = completely

TABLE 3 - Bone Graft Materials

Form of Bone Matrix	Type of Bone Matrix (top/bottom layer)	Number Code for Sample
sliced	DBM	1
powdered	DBM/DBM	2
"	DVBM/DVBM	3
"	DVBM+BMP/DVBM+BMP	4
"	DBM/DVBM	5
"	DBM/DVBM+BMP	6
"	DVBM+BMP/DVBM	7

Sample Numbering Scheme
86 - 5025 - 109 - ___ - __ - ___ - _____

Animal Protocol Number
Bone Type Number Code
Sample Region (if cut on saw)
Experiment Number
Embedded Block Number

TABLE 4 - Cell Culture Numbering Scheme

APPENDIX 3

APPENDIX 3 - MATERIALS AND EQUIPMENT

A. Animals

20 Day Pregnant Sprague Dawley Rat

Rabbit Cortical Bone (Femur/Tibiae)

B. Chemicals

Azure A		
Benzoyl Peroxide		
Calcium Chloride	$CaCl_2$	In-house
Calcium Sulphate	$CaSO_4$	
Carbon Dioxide Gas	CO_2	In-house
Chicken Plasma	CP	Fisher
Chloroform	$CHCl_3$	Fisher
CMRL-1066 Medium	CMRL1066	Gibco
Dry Ice		
Eosin		
Ethanol	EtOH	Fisher
Ethyl Ether	Et_2O	In-house
Ethylenediamine Triacetic Acid	EDTA	Fisher
Fetal Bovine (Calf) Serum	FCS	Gibco
Hemotoxylin		
Hydrochloric Acid	HCl	In-house
Hydroxyapatite	HA	Orthomatrix
Lithium Chloride	LiCl	
Methanol	MeOH	Fisher
Methyl Methacrylate	MMA	Fisher
Methyl Salicylate	MS	Fisher

Neutral Buffered Formalin	NBF	
Neutral Phosphate Buffer	NPB	
Paraffin		
Penicillin-Streptomycin Antibiotic	P/S	Gibco
Permamount		Fisher
Phosphate Buffered Saline		
Potassium Permangante	$KMnO_4$	
Powdered Hank's Balanced Salt Solution	HBSS	Gibco
Sodium Bicarbonate	Na_2CO_3	In-house
Sodium Hydroxide	NaOH	In-house
Toluidine Blue O	TBO	
Uranyl Nitrate	$UO_2(NO_3)_2$	
Vitrogen	Vit	Collagen Corp
Wet Ice		
Xylene		

*Rabbit Tibia/Femurs (source of sliced & powdered DBM)

**20 Day Pregnant Sprague Dawley Rat (source of fetal skeletal muscle)

C. Equipment

Absorbant Pads (or Filter Paper) for Culture Dish Outer Well

Autoclavable Glass Bottles with Screw Caps

Autoclave

Automatic Rotator

Benchtop Hood with UV Lamp

Bone Mill

Dissecting Scope

Dissecting Tools

Fiber Optic Lamp

Glass Cover Slips

Glass Slides

Graduated Cylinders

Graduated Tubes with Screw Caps

Guillotine

Histomat

Hot Plate with Stirrer

Incubator

Laminar Flow Hood

Lyophilizer

Microscope

Miller

Paraffin Mold Frames

Paraffin Embedding Case

Pasteur Pipets

Petri Dishes (Assorted Sizes)

PH Meter

Polycut E Microtome

Refrigerator/Freezer

Sieve Grating

Staining Wells

Sterile Disposable Filter System

Sterile Graduated Pipets

Temperature Controlled Oven

Temperature Controller Water Bath

Volumeric Flasks

Water Deionizer Still

Welled Culture Dishes

APPENDIX 4

APPENDIX 4 - PREPARATION OF SOLUTIONS

A. Preparation of Solutions - Demineralization

1. 0.6N Hydrochloric Acid (HCl)

To a 500 millilter (ml) volumeric flask was added 300 ml of deionized distilled water (ddH_2O) and 9.12 ml (0.3 moles) of concentrated HCl. The solution was well mixed and diluted to 500 ml with ddH_2O. The 0.6N HCl solution was stored in a sterile glass bottle in the refrigerator.

2. 2.0M Calcium Chloride ($CaCl_2 \cdot 2H_2O$)

To a 500 millilter (ml) volumeric flask was added 300 ml of deionized distilled water (ddH_2O) and 147.0 grams (1.0 moles) of calcium chloride dihydrate. The solution was well mixed and diluted to 500 ml with ddH_2O. The 2.0M $CaCl_2$ solution was steriled using a Corning disposable filter system and stored in the refrigerator.

3. 0.5M Ethylenediamine Triacetic Acid (EDTA)

To a 500 millilter (ml) volumeric flask was added 300 ml of deionized distilled water (ddH_2O) and a solution of 73.06 grams (0.025 moles) of ethylenediamine triacetic acid in 300 ml of $dd_{H2}O$ (which had been adjusted to pH 7.4 via NaOH pellets over an ice bath). The solution was well mixed and diluted to 500 ml with ddH_2O. The 0.5M EDTA solution (pH ~7.4) was steriled using a Corning disposable filter system and stored in the refrigerator.

4. 8.0 Lithium Chloride (LiCl)

To a 500 millilter (ml) volumeric flask was added 300 ml of deionized distilled water (ddH_2O) and 169.56 grams (4.0 moles) of lithium chloride. The

solution was well mixed and diluted to 500 ml with ddH_2O. The 8.0M LiCl solution was steriled using a Corning disposable filter system and stored in the refrigerator.

B. Preparation of Solutions - Cell Cultures

1. Fetal Calf Serum - Heat Activated

The fetal calf serum was stored in the freezer at -20^{o}C as purchased from the manufacturer. To heat activate the serum, the bottle was removed from the freezer and thawed in the refrigerator for 2-3 days. The loosely capped bottle was placed in a water bath which was temperature controlled to 55-58 ^{o}C for 30 minutes. The warm, haet activated fetal calf serum was transferred to autoclaved graduated test tubes with screw caps in 16 milliliter portions per tube using sterile techniques. The tubes were dated and stored at -20 ^{o}C in the freezer until needed.

2. Hank's Balanced Salt Solution

A 1X solution can be purchased from a manufacturer or prepared from a powder (see below procedure).

To a 500 milliliter volumeric flask was added 400 milliliter deionized distilled water (ddH_2O), followed by the addition of the entire contents of one package (9.8 grams per package) of powdered Hank's Balanced Salt Solution (HBSS)* at romm temperature (RT). If necessary the inside of the package was rinsed with small amounts of ddH_2O. The solution was mixed well, then 0.175 grams of sodium bicarbonate ($NaHCO_3$) was added. This mixture was diluted to 500 milliliters with ddH_2O. The pH of the solution was adjusted to 7.1-7.2 using 1N HCl or 1N NaOH. A note from the manufacturer pointed out that sterile filtration will increase the solution pH to the desired pH of 7.4. Sterile filter solution and store the 1X HBSS (pH 7.4) in the refrigerator until needed.

*Note: The 9.8 gram package of powdered HBSS is stored in the freezer and removed as needed.

3. CMRL-1066 Culture Medium

The CMRL-1066 culture medium was prepared within 24 hours of the cell culture experiment.

To an autoclaved 100 milliliter graduated cylinder was added 81.5 milliliters (81.5 volume %) of 10X concentrated CMRL-1066 medium, 16 milliliters (~16 volume %) of heat activated fetal calf serum, 3.0 milliliters of 0.7 M sodium bicarbonate solution via sterile pipet and 0.5 milliliters (0.5 volume %) of penicillin-streptomycin antibotic 10,000 units/ml and 10,000 micrograms/ml, respectively, via sterile pipet. The CMRL-1066 medium was sterilezed by disposable sterile filter system, then refrigerated between usage.

4. Vitrogen

A 10 millilter portion of neutralized, isotonic vitrogen solution was prepared on the same day as the cell culture experiment.

To an autoclaved vial was pippeted 8.0 milliliters of cold vitrogen 100 collagen, 1.0 milliliters of sterile 0.1M NaOH and 1.0 millilters of 10X sterile phosphate buffered saline solution, using sterile conditions. The pH of this solution was adjusted by the dropwise additon of 0.1 M NaOH or 0.1 M HCl until the pH equaled 7.4 +/- 0.2. The neutralized, isotonic solution was stored in the sterile hood at room temperature for gelation. Note: gelation occurs within two to three hours at room temperature. If necessary, the capped vial ccould be placed on a warm water bath to enhance the gelation.

C. Preparation of Solutions - Fixation, Infiltration, Embedding

1. Neutral Buffered Formalin (NBF)

To a 1000 milliliter (ml) volumeric flask was added 500 ml of distilled deionized water (ddH_2O), 4.0 grams of sodium phosphate monobasic, 6.5 grams of sodium phosphate dibasic anhydrous and 10 ml of 37-40% aqueous formaldhyde. The solution was well mixed and diluted to 1000 ml with ddH_2O. The pH of the solution was adjusted to 7.0 +/- 0.1 using NaOH or HCl.

2. 100%, 95%, 85% and 70% Ethanol (EtOH)

To a 500 milliliter (ml) volumeric flask was added 500 ml, 475 ml, 425 ml or 350 ml of 200 proof ethanol then diluted to 500 ml with distilled deionized water (ddH_2O) to give a 100%, 95%, 85% and 70% solution of EtOH, respectively.

3. Dry Benzoyl Peroxide

To a beaker of 100 milliliters (ml) of 200 proof ethanol was added 5-10 grams of wet benzoyl peroxide (as shipped from the manufacturer) under the hood. The slurry was carefully stirred for 5-7 minutes. The ethanol was removed by vacuum filtration to recover the"dry" material. The material was air dried for 15 minutes before storing in a closed metal container under the hood. Note the dry material can ignite, thus caution should be used during handling.

4. Soft Methyl Methacrylate (MMA) I, II and III

a. Soft MMA I

A solution of 75 milliliters (ml) of methyl methacrylate and 25 ml of n-butyl phthalate was stirred for 3 hours. The solution was used immediately.

b. Soft MMA II

A solution of 75 milliliters (ml) of methyl methacrylate, 25 ml of n-butyl phthalate and 1.0 grams of dry benzoyl peroxide was stirred for 3 hours. The solution was used immediately.

c. Soft MMA III

A solution of 75 milliliters (ml) of methyl methacrylate, 25 ml of n-butyl phthalate and 2.5 grams of benzoylperoxide was stirred for 3 hours. The solution was used immediately.

5. Hard Methyl Methacrylate (MMA) III

A solution of 100 milliliters of methyl methacrylate and 2.5 grams of benzoyl peroxide was stirred for 3 hours. The solution was used immediately.

D. Preparation of Solutions - Glass Slides

1. Chromerge Solution

A solution of 5.0 milliliters of chromerge and 500 milliliters of concentrated sulfuric acid was mixed well and stored under vented hood.

2. Gelatin Alum Solution

The gelatin solution was prepared by adding of 4.5 grams of unflavored gelatin powder in 1000 milliliters of distilled water. This suspension was heated to 75^{o}C until the solution was homogeneous.

The chromium alum solution was prepared by adding 1.54 grams of chromium potassium sulfate to 38.5 milliliters of distilled water.

The gelatin (1000 milliliters) and chromium alum solutions (38.5 milliliters) were mixed together and heated to 60^{o}C with stirring. The solution is stored in the refrigerator and reheated when needed.

E. Preparation of Solutions - Staining

1. Periodic Acid Schiff (PAS) - Alcian Blue

a. Schiff's Reagent

A strongly acidic solution of 3.0 grams of basic fuchsin in 90 milliliters of concentrated hydrochloric acid was carefully added to a solution of 0.5 grams of metabisulfite ($Na_2S_2O_5$) in 510 milliliters of distilled water. The solution was transferred to a brown bottle with screw cap and allowed to sit at room temperature for 24 hours. Activated characoal was added to decolor the solution, then the solution was filtered. The colorless solution was stored in the refrigerator.

b. Mayer's Hemalum

To a solution of 0.5 grams of hematoxylin in 500 milliliters of distilled water was added 0.1 grams of sodium iodate ($NaIO_3$) and 25.0 grams of aluminium potassium sulphate. The solution was transferred to a brown bottle with screw cap and stored at room temperature.

c. Alcian Blue

To a solution of 500.0 milliliters of 3% acetic acid was added 5.0 grams of Alcian Blue. The solution was stored at room temperature in a brown bottle with a screw cap.

APPENDIX 5

APPENDIX 5 - EXPERIMENTAL PROCEDURES

A. Demineralization of Bone

1. Rabbit Bone

The cortical bone from rabbit tibiae and femurs were harvested immediately (after sacrifice) then cleaned of all blood vessels and tissues storing temporarily in cold saline. The cleaned bone was chunked (cut) into small strips and stored in the freezer until sufficient material was on hand for demineralization. Two techniques were used for demineralizing the rabbit bone, however the latter procedure was chosen as the standard for this experiment. It should be noted that the harvested bone must be kept at temperatures below $0^{o}C$ to prevent the reduction of the protein activity. The solutions were prepared with sterile distilled deionized H_2O then stored at $2^{o}C$. All extractions were performed under a 1:100 bone weight to liquid volume ratio.

a. Method I

The cortical bone was placed into 0.6N HCl for 24 hour periods under refrigeration. The solution was changed daily until the bone appeared very pliant and translucent. The demineralized bone was washed with copious amounts of sterile distilled water for 48 hours under refrigeration then placed in 100% EtOH for 24 hours. Followed by washing in ethyl ether for 24 hours. The demineralized bone was lyophilized for 24 hours and stored in the freezer until needed.

b. Method II (Nathanson et al, 1978)

The cortical bone was placed into 1:1 chloroform to methanol mixture (by volume) at room temperature for 2hrs (sliced DBM) or 1hr (powdered DBM) then washed with sterile dH_2O at $2^{o}C$ for 0.25 hrs. The material was demineralized with

0.6N HCl at 2°C for 72hrs (sliced DBM) or 24hrs (powdered DBM) then washed with sterile dH_2O at 2°C for 0.25 hrs. A 2.0M $CaCl_2$ solution was used to remove proteins and polysaccharides at 2°C for 2hrs (sliced DBM) or 1hr (powdered DBM) then washed with sterile dH_2O at 2°C for 0.25 hrs. A 0.5M ethylenediamine triacetic acid (EDTA) solution was used at pH 7.4 to remove residual proteins at 2°C for 2hrs (sliced DBM) and 1hr (powdered DBM) then washed with sterile dH_2O at 2°C for 0.25 hrs. A 8.0M LiCl solution was used as a denaturant at 2°C for 2hrs (sliced DBM) or 1hr (powdered DBM) then washed with sterile dH_2O at 2°C for 0.25 hrs and warmed in deionized dH_2O at 55°C for 2hrs (sliced DBM) or 1hr (powdered DBM). The demineralized bone was lyophilized and stored at -70°C.

c. Method III (Essex Medical Products, Wheat Ridge, CO) [Damien (1989)]

The powdered cortical bone was demineralized in 20 milliliter of 1M hydrochloric acid per gram of powdered bone at 4°C for 16 hours. Half of the demineralized bone matrix (DBM) was stored in the freezer. The other half was used to prepare devitalized bone matrix.

The powdered DBM was treated with 20 milliliters of 4M guandine hydrochloride (buffered to pH 7.4 with 0.1 M tris) per gram of powdered cortical bone at 4°C for 16 hours. This devitalized bone matrix (DVBM) was stored in the freezer.

2. Bovine Bone (Essex Medical Products, Wheat Ridge, CO) [Damien (1989)]

The cortical bovine bone was demineralized according to the same procedures described above for rabbit bone-Method III. The demineralized bone was extracted with 4M guandine hydrochloride then purified by a series of chromatographic techniques. The resulting non-collagenous protein fraction showed osteoinduction activity. This activity was confirmed in Essex Medical

Products' subcutaneous implant model prior to shipment. The bone morphogenetic protein (BMP) was stored in the freezer.

B. Preparation of Fetal Rat Skeletal Muscle

Twenty day pregnant Sprague-Dawley rats were sacrificed by guillotine or CO_2 gas chamber. The fetuses were removed from both horns of the uterus and immediately placed into a sterile petri dish containing freshly prepared Hank's balanced salt solution. The fetal sacs were removed and the fetuses were cleaned of blood. They were transferred into another dish of Hank's solution where the heads were removed. The skin of each hind leg was removed and the leg was cut to include as much of the thigh muscle and femur as possible. The legs were placed into freshly prepared, chilled CMRL 1066 medium. The skin, cartilage, bone, connective tissue, blood vessels and other nonmuscular tissue were stripped off to obtain clean muscle. The muscle was further inspected and teased into small packets. The bundles of muscle were placed into fresh CMRL 1066 medium and put on ice until combined with the rehydrated demineralized bone matrix, devitalized bone matrix and devitalized bone matrix plus bone morphogenetic protein in the absence and presence of hydroxyapatite.

C. Preparation of Bone Matrix-Cell Cultures

1. Sliced DBM

The vial containing the sterile lypholized sliced DBM was removed from the freezer and put on ice at the beginning of the experiment. The pieces of DBM were cut into smaller slices (about 5 mm in length) and five to six longitudinal notches were made along both sides of the slices. Thirty minutes prior to combining with the bundles of fetal skeletal muscle, the slices were placed in a sterile petri dish using only the estimated amount needed for the experiment. Approximately thirty six slices of DBM were used per pregnant rat (two pieces were needed per culture). A sufficient amount of chicken plasma was added to the dishes to rehydrate the lypholized frozen slices of DBM using sterile techniques.

2. Powdered DBM, DVBM and DVBM+BMP

The vials containing the sterile lypholized powdered DBM, DVBM and DVBM+BMP (material supplied from Matrix) were removed from the freezer and put on ice at the beginning of the experiment. Thirty minutes prior to combining with the bundles of fetal skeletal muscle, the powdered DBM, DVBM and DVBM+BMP were individually placed in sterile petri dishes using only the estimated amount needed for the experiment. A sufficient amount of chicken plasma was added to the dishes to rehydrated the lypholized frozen bone matrix powders using sterile techniques.

3. Powdered DBM and DVBM+BMP with HA

The vials containing the sterile lypholized powdered DBM and DVBM+BMP were removed from the freezer and put on ice at the beginning of the experiment. Thirty minutes prior to combining with the bundles of fetal skeletal muscle, the powdered DBM and DVBM+BMP were individually placed in a weighed sterile vial.

After determining the amount of respective bone matrix material in each vial, HA was added to the vials under sterile conditions until a ratio of 3:1 DBM/HA was established. A sufficient amount of chicken plasma was added to the composite material to rehydrate the lyophilized frozen bone matrix powders using sterile techniques.

D. Preparation of Cell Culture

The double welled culture dishes were prepared using sterile handling techniques in a laminar flow hood or a benchtop hood with UV lamp. In the outer well of each culture dish was placed a sterile dehydrated ring and a triangular shaped wire mesh "shelf" was placed over the inner well. Sterile distilled deionized water was used to thoroughly hydrate the ring in the outer well of each dish. Fresh solutions of 100 ml CMRL-1066 medium and 5 ml vitrogen was prepared. The freshly prepared CMRL-1066 medium, chicken plasma and bone matrix material(s) were placed on ice. The vitrogen solution was placed inside the sterile hood at room temperature to allow gelation to occur.

1. Sliced Demineralized Bone Matrix

The sliced DBM which has been previously hydrated with chicken plasma is placed on the wire mesh, only three slices per culture dish was used. The canals in the sliced DBM were filled with the packets of teased fetal rat skeletal muscle. Any excess chicken plasma from the DBM or excess CMRL-1066 medium from the skeletal muscle was pipetted out of the canal and another piece of hydrated sliced DBM was placed on top of the muscle. The inner well in the culture dish was filled with the freshly prepared CMRL-1066 medium until a small pool of the medium formed beneath each DBM/muscle/DBM culture sample. The cultures were placed in an incubator at 37.5^{o}C and purged with CO_2. The air flow was maintained at 4.75 liters/min, CO_2flow at 0.25 liters/min and the H_20 level jacketing the chamber was kept midway between the high and low markings on the indicator. Note for additional humidity inside the chamber of the incubator, a glass dish was filled with distilled dionized H_2O. If the incubator did not have the feature of an outer water jacket, the water inside this glass dish was checked every two to three days and the temperature of the cell culture room was kept as constant as

possible to minimize any drop in temperature whenever the incubator door was opened.

2. Powdered Bone Matrix Material(s)

The partially gelatinized vitrogen, a collagen solution, which has been setting for two to three hours was placed on the wire mesh "shelf" in it's lumpy form. The vitrogen was transferred dropwise via a sterile pipet until approximately a 10mm circular bed was formed on the wire mesh shelf. Note that only one sample was placed in each culture dish. Small amounts of the powdered DBM, powdered devitalized bone matrix (DVBM) or powdered DVBM plus bone morphogenetic protein (DVBM+BMP) that have been hydrated with the chicken plasma was carefully sprinkled on top of the vitrogen bed. A microscope was used to visually ensure that the bone matrix material was evenly covering the vitrogen. Packets of the teased fetal skeletal muscle was placed on top of the powdered bone matrix material. Any excess chicken plasma and CMRL-1066 medium was pipetted off and an additional amount of the hydrated powdered bone matrix material was sprinkled on top of the muscle. The inner well was filled with the fresh CMRL-1066 medium and the powdered bone matrix/muscle/powdered bone matrix culture samples were placed in the incubator using the same incubator settings as described in the above section. The vitrogen bed will finish the gelation process at the 37.5^{o}C incubator temperature.

E. Maintenance of Cell Cultures

Every two days, the CO_2 tank pressure, the H_2O outer jacket level and H_2O level in the glass dish inside the incubator were checked. At the same time, fresh CMRL-1066 medium that was stored in the refrigerator was warmed on a water bath to 38-40^oC for 15-20 minutes. The laminar flow hood or benchtop hood was wiped down with 70% ethanol then allowed to sit with the laminar flow air system or UV lamp on respectively for 30-60 minutes to sterilize the hood. The shelf in the incubator containing the culture dishes was carefully removed from the incubator and placed in the sterile hood. With sterile disposable glass pipets, approximately 50-75% of the medium in the inner well of the culture dishes were removed with care not to contaminate the cells. Fresh warm CMRL-1066 medium was added until a small pool of medium formed beneath the culture(s) on the wire mesh shelf. It should be noted that a new sterile pipet must be used for each culture dish such that the potential for cell contamination is reduced. A dramatic color change in the medium is noted during the first 2-3 medium changes (the first week). The hydrated ring in the outer well of the culture dish was checked for the degree of wetness and if necessary, additional amounts of sterile warm distilled deionized H_2O was added until the ring was thoroughly wet. The shelf was returned to the incubator and the chamber was purged with CO_2 for 1 minute.

This maintenance procedure was repeated every two days until approximately 16-20 days elapsed. Several days prior to the termination of the cultures, 5-7 drops of freshly prepared, partially gelatinized vitrogen was added to the top of the cultures containing the powdered bone matrix via sterile pipet. This top layer and coating of vitrogen was used to hold the particles of the bone matrix together during the histological processes of fixation and embedding. These vitrogen coated cultures were returned to the incubator and the experiment was terminated 2-3 days later.

F. Preparation of Cultures for Plastic Embedding

1. Fixation Procedures

The cell cultures were removed from the incubator on the day of termination of the experiment. Each culture was carefully placed into a separate vial using sterile forceps or a scoopula. Note that for the transfer of the cultures consisting of the powdered bone matrices, the gelatinized vitrogen layer was carefully separated from the wire mesh shelf using a sterile flat spatula or scoopula. The cultures were washed with neutral phosphate buffer (NPB), using approximately 10 times the volume of the specimen, for 5 minutes at room temperature (RT). The solution was decanted out of each vial. The cultures were suspended in 10% neutral buffer formalin (NBF) for 24 hours with occasional swirling at RT then the solution was decanted off.

2. Dehydration Procedures

The cultures were dehydrated by treatment with 40% ethanol for 3 hours followed by treatment with 70% ethanol for at 3 hours at RT. Note that if immediate storage was necessary, the specimens could be kept in a 70% ethanol solution at RT. However for better histological results the specimens should be completely fixed and embedded.

To ensure optimal dehydration, the vials were placed on a automatic rotator after each of the following steps. The 70% ethanol solution was decanted off then the samples were treated with 85% ethanol for 2 hours and 16 hours (fresh solution used), twice with 95% ethanol for 2 hours, and two washing with 100% ethanol (200 Proof) for 16 hours and 2 hours. In order to remove the ethanol from the specimens and to allow the complete infiltration of the methylmethacrylate, the specimens were immediately suspended twice in xylene (or hemostat which is not as toxic as xylene) for 2 hours.

3. Infiltration Procedures

The samples were treated with three different concentrations of the soft methyl methacrylate solutions, referred to as sMMA I, II and III. This gradual increase in the concentration of the MMA allows the samples to become fully infiltrated with the embedding solution of sMMA III. Each sMMA solution is prepared approximately three hours before use.

The xylene was immediately decanted off and the samples were treated with soft MMA I for 1-3 days. Note that the sample should not stay in any MMA solution for more than 1-2 days without constant checking otherwise the samples may start to polymerize in the wrong MMA solution. The soft MMA I was decanted off then the samples were treated with soft MMA II for 24 hours and soft MMA III for 24 hours.

4. Embedding Procedures

The square glass vials were prepared for later use by adding approximately 5 milliliters of hard MMA III to each vial. Note one vial is needed per sample. The vials were sealed with the screw caps and placed on a level water bath for 3-5 days at 37^{o}C. The MMA layer was checked daily for hardness.

The samples were transferred to a square shaped glass vial fitted with a screw cap. Inside each of the vials was placed a layer of polmerized hard MMA III. The samples were embedded inside these glass vials using freshly prepared soft MMA III. Care was taken to orient the samples inside the vials before polmerization began. The vials were returned to a water bath to catalyze the polmerization. Note that the temperature of the water bath is critical. The polymerization reaction gives off heat, therefore if the bath is kept too hot the MMA will heat too quickly and cause bubbles to form in the MMA (plastic) surrounding the sample.

The completing of the polymerization was checked by testing the firmness of the MMA. Complete polymerization is marked by the firmness of the top surface of the MMA. Once the entire volume of the MMA was polymerized, the samples were labelled according to the assigned protocol number (86-5025-109), the experiment number, type of bone matrix, block number and region of cell culture [see Table 4].

G. Preparation of Embedded Samples for Sectioning

The cell cultures embedded in polymerized MMA were cooled in the freezer for 1 hour then removed from the glass vials by breaking the walls of the vial with a hammer. The sample blocks were trimmed to a smaller size. Note the base of the blocks were kept in tact but some of plastic surrounding the sample was remove so that the cross sectional cuts would have less surface area.

H. Preparation of Glass Slides for Sectioning

Glass slides (precleaned from the manufacturer) were cleaned with fresh chromerge solution by soaking the slides for 5 minutes in the solution. Note that the slides were mounted in a glass rack during the cleansing process. The slides were rinsed thoroughly in three consecutive distilled water baths. The slides were transferred to a slides storage box via forceps and air dried overnight.

The gelatin-chromium alum working solution [see Appendix 4] was removed from the refrigerator, warmed to room temperature then heated to $50^{o}C$ on a stirrer hot plate. The clean dry slides were transferred to a glass rack and submerged in the warmed gelatin solution for 2 minutes to coat the slides. The slides were transferred to the slide storage box for drying via forceps. This thin coating will help the microtomed sections of the embedded sample to adhere to the slides.

I. Microtome Sectioning of the Embedded Samples

The trimmed samples were placed in a Reichert-Yung Mircotome. A block from each bone matrix type was sliced in 80-100 serial sections at a thickness of 5 microns. Four slices were mounted on each coated glass slide as they were sectioned. Each slide was wrapped in clear plastic wrap to smooth the slices, the slides were stacked on top of one another, plexiglass spacers were added to the top and bottom of the stack, the stack was carefully clamped and the slides were placed in an oven at $60^{o}C$ for 18 hours to assure the smooth adhesion of the slices to the slide.

J. Preparation of the Embedded Samples for Regional Sectioning

After 80-100 serials sections were sliced on the microtome, an embedded sample, representing each bone matrix type, was cut into 3-4 segments using a diamond. Each segment was cut parallel to the last serial section at a thickness of 2 millimeters. The number of segments for each sample was dependent upon the diameter of the vitrogen/bone matrix bed of the culture and the number of serial sections sliced. Thus each segment (A-D) will provide information about 3-4 regions of the culture. Note for a sample with three segments: region A is the region closest to the initial serial sections, region B is near the center of the sample and region C is the other end of the sample [see Figure 12].

Each segment was carefully labelled for the correct spatial orientation, re-embedded, prepared for sectioning and sectioned according to procedures described in the Embedding Procedures, Preparation of Embedded Samples for Sectioning and Microtome Sectioning Procedures.

K. Staining of Glass Slides

The slides were deplasticized in two methyl acetate baths for 15 minutes. The sections were hydrated in consecutive ethanol baths of 100%, 95%, 80%, 70%, 40%, 20% for 1 minute followed by distilled water bath for 1 minute and 3% acetic acid bath for 3 minutes.

The slides were stained with the Alcian Blue/Acetic Acid solution [see Preparation of Solutions, Appendix 4] for 2 hours followed by washing in running tap water for 5 minutes. The samples on the slides were oxidized with 0.5% Periodic Acid solution for 30 minutes followed by rinsing in tap water.

The slides were stained with the Schiff's Reagent [see Preparation of Solutions, Appendix 4] for 30 minutes followed by washing in running tap water for 5 minutes. The slides were stained in Mayer's Hemalum [see Preparation of Solutions, Appendix 4] for 10 minutes followed rinsing in distilled water.

The staining of the slides was differentiated with warm tap water. The samples were dehyrated by passing the slides through a series of consecutive ethanol baths (30% ---> 100%). The slides were cleared in xylene and mounted with cover slip for examination under light microscopy.

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