Elution Characteristics of Bone Morphogenetic Proteins from

Demineralized Bone Matrix

BY

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THESIS

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I would like to dedicate this thesis to my husband, Muhammad Ali, whose love and moral support has always encouraged me in achieving my goals.

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LIST OF ABBREVIATIONS

FDA   Food and Drug Administration

AATB   American Association of Tissue Banks

MSC   Mesenchymal Stem Cells

HSC   Hematopoietic Stem Cells

ALP   Alkaline Phosphatase

BMP   Bone Morphogenetic Protein

rhBMP   Recombinant Human Bone Morphogenetic Protein

TGF-β   Transforming Growth Factor Beta

GuHCl   Guanidine Hydrochloride

HCl   Hydrochloric Acid

DBM   Demineralized Bone Matrix

ELISA   Enzyme-Linked Immunosorbent Assay

SB   Sorenson's Buffer

PI   Protease Inhibitors

CD   Collagenase Digestion

GPS   Graft Preparation System

BDBM   Bonus Demineralized Bone Matrix

SUMMARY

Little is known of the elution characteristics of bone morphogenetic proteins (BMPs), proteins involved with the bone-healing cascade, out of demineralized bone matrix (DBM). DBM is a type of graft commonly used for in-vivo orthopedic surgical treatments to repair various bone abnormalities. Knowledge of the elution characteristics of BMPs out of DBM could improve the understanding of DBM function and help optimize graft design.

The elution characteristics of BMPs out of DBM were examined in-vitro for a period of 3 months using a variety of approaches as a probe to determine the association of BMPs with DBM over time. Results suggested that once the DBM is placed into an aqueous environment, a rapid elution of BMPs out of DBM takes place, followed by a slow prolonged release of BMPs. However, as the DBM hydration time increases, there is an unexpected increase in DBM BMP content as well; for example, more BMPs in the DBM can be accounted for 7 days into DBM hydration versus 0 hours of DBM hydration. Because obviously new BMPs are not spontaneously created, results support the idea that BMPs can be loosely or tightly associated with DBM which allows for BMPs to elute out of DBM dependent on DBM hydration time; for example, the loosely bound BMPs would initially quickly elute out of DBM, whereas the tightly bound BMPs would slowly be released, creating a BMP reservoir for the bone forming mechanism.

I. INTRODUCTION

A. BACKGROUND

1. Medical Demand

The number of orthopedic procedures performed is rising. An estimated one million bone-grafting procedures are performed globally every year in attempts to repair or reconstruct bone in various parts of the body. The clinical use of bone grafts is predicted to steadily grow due to an aging population, an increase in the number of revision orthopedic surgeries, and a rise in the number of seniors seeking an active lifestyle (1).

Bone grafts are used for a variety of orthopedic surgical treatments, some of which include the repair of complex bone fractures (2), fusion of bone structures such as vertebral bodies in order to correct spinal deformities (3), filling voids due to intentional removal of bone such as bone tumors (4), providing structural support or replacing bone loss during surgical procedures such as joint arthroplasty (5,6), as well as cosmetics and dentistry for procedures such as tooth implants (7).

2. Bone

2.1 Bone Structure and Composition

The structure of bone is ideal for load bearing while working in concert with muscle to provide motion for proper function. Bone as a tissue consists of two main types: woven and lamellar bone (8). Woven bone is characterized by the presence of randomly oriented

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collagen fibers and is seen in the bones of young children. It is the first tissue to appear in the process of bone healing (described in detail below). Lamellar bone, on the other hand, is also known as mature bone. It is characterized by the presence of collagen fibers arranged in parallel layers or sheets (lamellae). Lamellar bone is present in both structured types of bone, cortical (compact) bone and cancellous (trabecular or spongy) bone.

Cortical bone mainly consists of the osteon and periosteum. The osteon is composed of a central opening containing blood vessels and neural tissue; as well as cylindrical tubes made of concentric lamellae. Although the orientation of collagen fibers within the same lamella are predominantly parallel to one another, the orientation of the fibers may change up to 90 degrees in adjacent lamellae, which provide torsion and bending strength (8,9). The periosteum is a fibrous tissue covering which enables attachment of muscles and tendons to bone, and is also highly osteogenic which aids in bone healing. The light and spongy cancellous bone is usually surrounded by cortical bone, which provides greater strength and rigidity. The porous structure of cancellous bone enables it to dampen sudden stresses, as in load transmission through joints. It is organized into a three-dimensional lattice work of bony processes called trabeculae which are oriented in the direction of the loads to maximize strength and minimize mass (Wolff's Law). The combination of vertical and horizontal trabecular struts prevents the bone from bowing. The pores of the cancellous bone are filled with bone marrow, the soft and fatty vascular tissue involved with the production of red and white blood cells.

Compositionally, bone consists of mineral, organic, and aqueous phases. More specifically, these phases are hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$); organic matrix which consists of mainly type I collagen fibrils (> 90%) and noncollagenous proteins such as proteoglycans and phospholipids; and water, which is associated with the intra- and extracellular components of the structure, respectively. Pietrzak, et al (10) measured the weight composition of the various phases of cleaned and dried human cortical bone obtained from 20 donors that had passed Food and Drug Administration (FDA) and American Association of Tissue Banks (AATB) donor screening procedures. They found mineral, bone matrix (predominantly Type I collagen), and lipid contents of 67.0±1.3 wt%, 31.9±1.1%, and 1.1±1.5%, respectively.

For a cellular breakdown, bone is composed of osteoblasts, osteoclasts and osteocytes. Osteoblasts, the bone forming cells, are derived from mesenchymal stem cells (MSCs – multipotent stem cells isolated from the mesenchyme, or embryonic connective tissue, that can differentiate into a variety of cell types) that line the surface of the bone and produce osteoid, the unmineralized, organic portion of bone that forms before bone tissue matures. Osteoclasts are responsible for bone resorption, or the breakdown of bone; they are derived from hematopoietic stem cells (HSCs - cells isolated from blood or bone marrow that renew themselves or can differentiate to a variety of specialized cells) and reside in bone resorption pits on the bone's surface called Howship' s lacunae. Osteocytes are mature bone cells; when osteoblasts become trapped in the matrix they secrete, they become osteocytes (9).

2.2 <u>Bone Healing</u>

Bone develops in two different ways, either by intramembranous or endochondral ossification; the essential difference between them is the absence or presence of a precursor cartilaginous phase, respectively. Intramembranous ossification is the method by which bone grows in width, and therefore mainly responsible for the formation of the thin, flat bones (such as some of the bones of the skull and collarbones); it is initiated by the transformation of MSCs directly into osteoblasts to form bone. Endochondral ossification is the mechanism by which bone grows in length, and therefore mainly responsible for the formation of long bones (such as the tibia and humerus); it does not directly start forming bone from MSCs but requires a cartilage precursor (9).

Whether healing of bone or soft tissue, the general sequence of inflammation, proliferation, and remodeling applies; however, bone is the only tissue that heals without scar formation. The following is a schematic representation of the time course of the various stages of bone healing (**Figure 1**) and a detailed description of the stages of the bone healing mechanism (9, 11):
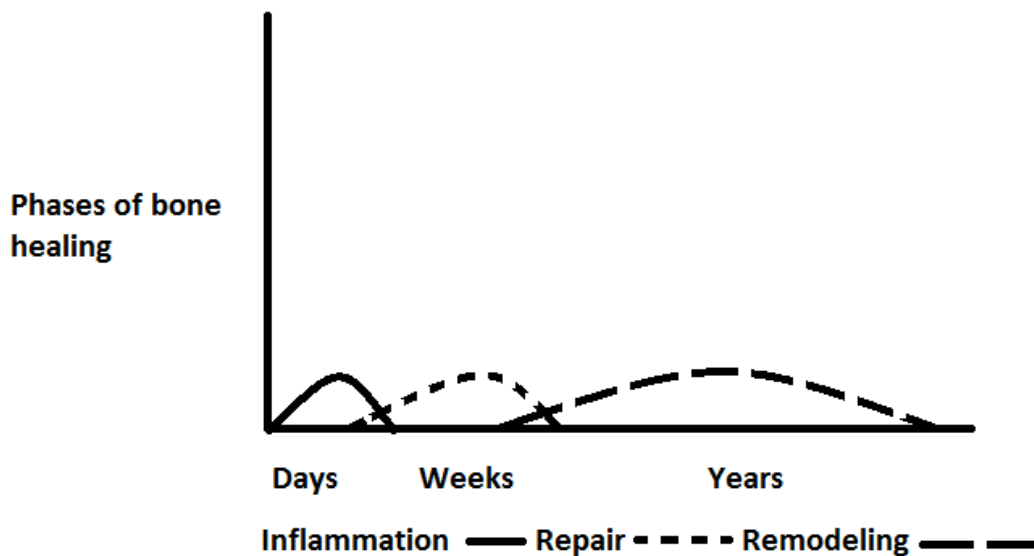
Figure 1. Schematic representation of the time course of the various stages of bone healing

Inflammatory Stage:

I. Induction (immediately)- Tissue disruption immediately results in a hematoma (blood clot) at the fracture site, which triggers the release of growth factors and cytokines.

Growth factors are naturally occurring biologic factors such as hormones or proteins that are released from platelets (fragments of precursor cells), macrophages (white blood cells that ingest foreign material such as infectious microorganisms), and fibroblasts (cells that synthesize the extracellular matrix and collagen, the structural framework for animal tissues, or stroma) to act as signaling agents for other cells involved in bone formation and healing by stimulating cell division, matrix synthesis, and tissue differentiation. Cytokines, on the other hand, are different from growth factors because they do not always have a positive effect on

cell division; while some cytokines can act as growth factors and promote cellular growth and proliferation, others have an inhibitory effect on cell growth.

II. Inflammation (first two days)- Inflammatory cells (macrophages, monocytes, lymphocytes, and polymorphonuclear cells) and fibroblasts invade the bone with the help of prostaglandin (hormone-like substance that stimulates target cells into action) mediation. This results in the ingrowth of vascular tissue and migration of MSCs. The sources of MSCs are bone marrow, periosteum, and surrounding soft tissues.

Repair Stage:

III. Cartilage formation (day 2 to 18) – Mitosis (multiplication of cells by division) of MSCs and differentiation to chondrocytes (cells that produce cartilage) takes place followed by the hypertrophy and cell death of chondrocytes.

IV. Woven bone formation (day 10 to week 16) – As vascular ingrowth progresses, the extracellular collagenous matrix is laid down by fibroblasts. Osteoblasts differentiate and secrete osteoid, subsequently mineralization of the extracellular matrix takes place. Mineralization occurs when osteoblasts secrete vesicles containing the enzyme alkaline phosphatase (ALP) which cleaves or liberates the free phosphate groups and acts as the deposition site for calcium and hydroxyapatite crystals to grow on.

Remodeling Stage:

V. Lamellar bone formation (months to years) - The remodeling of bone occurs slowly over months to years with the help of mechanical stress placed on bone and the woven bone is gradually converted to lamellar bone. Initially, resorption of bone takes place with the help of osteoclasts, followed by bone remodeling, formation of lamellar bone, and finally hematopoietic marrow. As the fracture site is exposed to an axial loading force, bone is generally laid down where needed and resorbed from where it is not needed (Wolff's Law). Satisfactory strength is usually achieved within three to six months.

3. **Protein Structure and Composition**

The human body is made of cells and each cell has a specific function; furthermore, each cell has several different proteins that make the cell function. Proteins are biochemical compounds that are designed to bind to simple or complex molecules. They allow a diverse range of biochemical reactions to occur through enzymatic catalysis, provide cell structure rigidity, control material flow through membranes, act as sensors and switches, cause motion, and control gene function. Proteins are constructed from 20 different amino acids that can be arranged in millions of different ways to create millions of different proteins, each with a specific function in the body. A single linear chain of two or more amino acids (monomers) attached by peptide bonds is called a polypeptide, and a protein consists of one or more polypeptide typically folded into a globular structure.

Following is a description of the general structure of amino acids, the building blocks of proteins. The center alpha carbon is bonded to four different chemical groups: an amino (NH_2) group, a carboxyl (COOH) group, a hydrogen (H) atom, and one variable group, called

a side chain or R group. All of the 20 different amino acids have this same general structure, but their side-chain groups vary in size, shape, charge, hydrophobicity, and reactivity. A single chemical linkage, the peptide bond, connects amino acids into a linear, unbranched chain. The peptide bond is formed by the condensation reaction (water-liberating) between the amino group of one amino acid and the carboxyl group of another. Therefore, at opposite ends of the chain a free, or unlinked, amino group (the N-terminus) and a free carboxyl group (C-terminus) exist. A typical mature protein structure is in globular conformation with bends and knots at specific residues of the amino acid chain (12).

4. Bone Morphogenetic Protein

In 1965, Marshall R. Urist was the first to observe that there is a substance in bone that has the ability to induce new-bone formation. He observed new bone had formed after the implantation of demineralized bone in a muscle pouch in the rat (13). He later identified a protein responsible for this effect that took on the name of "bone morphogenetic protein" or BMP.

In 1988, Wozney was able to identify the genetic sequence of bone morphogenetic proteins which eventually led to the discovery of the various BMP isoforms, and has made it possible to produce genetically engineered recombinant human BMPs (rhBMPs) using DNA technology (14,15,16). With the identification of BMP-2 to BMP-18 to date, BMP isoforms (isomeric forms of the same protein with slightly different amino acid sequences) 2, 4 and 7 play a critical role in bone healing by stimulating mesenchymal-derived cells such as monocytes (white blood cells) and fibroblasts to differentiate into bone-forming cells (11).

4.1 <u>BMP Structure and Composition</u>

BMPs are a group of cytokines that are part of the Transforming Growth Factor beta (TGF-β) superfamily with significant roles in bone and cartilage formation, along with other roles such as formation of other tissues, for example, of the heart and kidney. They are water-soluble glycoproteins with a relatively low molecular weight (under 50 kDa) and diffuse very easily in the body fluids; for example, BMP-2 and 7 have a molecular weight of 18 and 49 kDa, respectively (17). Initially, BMPs are synthesized as prepropeptides of approximately 400-525 amino acids (18-20). Cleavage of the variable length pro-segment occurs prior to secretion. Secretion of the 100-140 amino acid C-terminal mature segment forms a dimer (21). They are mature and biologically active in either homo or heterodimer conformation, consisting of two similar or different polypeptides, respectively; heterodimers in cell cultures have been observed to induce higher yields of the osteogenic marker ALP. Mature BMP segments are all assumed to form a cysteine (amino acid) knot with six cysteine residues, and there may be an additional one to three cysteines (22).

BMPs are both morphogens and mitogens; they are the only growth factors that have the ability to transform connective tissue cells into osteoprogenitor cells and stimulate the multiplication of connective tissue cells, respectively. All other growth factors are only mitogens and have the ability to induce multiplication of cells but are not morphogens because they do not transform one cell type into another (22).

After demineralization of bone, results such as from Urist's study above, suggest that osteoinductive (ability to recruit and convert mesenchymal cells to bone-forming cells to

produce new bone even at extraskeletal sites) proteins are mainly associated with the organic phase of bone and that the mineral phase masks the organic matrix, which inhibits the ability of the organic matrix to interact with water and prevents protein elution from the organic matrix (23). In vitro studies also suggest that when the mineral phase is present, it prevents protein extraction from the organic matrix using various protein extraction methods such as 4 M guanidine-HCl or 6M urea (24). Although that may be true, a study executed by Sampath and Reddi implicated that these osteoinductive proteins are also associated with the mineral phase; results showed that 15% of total biological activity of bone induction was associated with the mineral phase (25).

4.2 BMP Signaling Mechanism

Bone morphogenesis is a sequential cascade that BMPs are involved with which is comprised of three key phases: chemotaxis (migration of cells), mitosis, and differentiation of mesenchymal cells initially into cartilage – producing cells with the subsequent mineralization of this matrix and its replacement by bone. The sequential cascade begins with the binding of plasma fibronectin, a cell adhesive protein, to implanted demineralized bone, allowing mesenchymal cell attachment and maximal proliferation. Chondrogenesis, hypertrophy of cartilage, angiogenesis, bone mineralization and remodeling finally takes place (17).

Once released from platelets, macrophages, or fibroblasts (11), the active BMPs initiate signaling from the mesenchymal-derived cell surface when they bind to and bring together type I and type II serine-threonine kinase receptors. Type I and II receptors may exist independently, and as complexes in the cell membrane. However, optimal activation occurs

as a complex where the type II receptor is the primary binding site of the ligand and upon its activation, phosphorylation of the type I receptor occurs, and signals are then propagated to downstream substrates. Various signaling pathways have been proposed to be activated by the ligand binding to the receptors. However, recently, the Smad family of protein substrates has been identified as the downstream effectors of the phosphorylated type I receptor [17]. There are eight different Smads. Smads 1, 5, and 8 are substrates for BMP receptors and phosphorylation of either one of them activates them to interact with common partner Smad 4. This complex enters the nucleus to activate and turn on BMP-responsive genes. There are also two inhibitory Smads, Smads 6 and 7, that normally reside in the nucleus and act as a relay to inhibit and turn off BMP receptor I mediated phosphorylation of Smads 1, 5 and 8, resulting in a homeostatic control. These signaling pathways have the ability to transform mesenchymal cells into bone-forming cells (26).

When demineralized bone is placed in vivo, it is believed that the BMPs diffuse from the bone matrix into the surround region, considered to be essential for osteoinduction to occur (27). This would set up a BMP concentration gradient which could permit the bone-forming precursor cells to chemotactically follow this gradient to its source, which would be another potential BMP signaling mechanism. The physiological relevance of this is that when bone heals, osteoclasts collect at the margins of the bone defect and secrete HCl which locally dissolves the bone mineral. The demineralized bone at the defect margins can then elute BMPs to initiate the body's osteoinductive response at the site of injury where it is needed.

5. **Bone Grafts**

Currently, orthopedic surgeons have several options for bone replacement with the following graft categories; autografts, allografts, synthetic grafts (calcium phosphate or calcium sulfate-based bone graft substitutes), or demineralized bone matrix (DBM) which is a subcategory of allografts (28). These grafts can be categorized based on the mechanism of their contribution to bone healing. They can be: (1) osteoconductive - graft functions as a scaffold for the attachment and proliferation of bone-forming cells, vascular ingrowth, deposition and calcification of bone matrix; (2) osteinductive (property mentioned earlier) - graft has the ability to recruit and convert mesenchymal cells to bone-forming cells to produce new bone even at extraskeletal sites; (3) osteogenic – bone-forming cells of a graft are capable of forming new bone on or about the graft. Each type of graft can function as a combination of the properties mentioned above (23). **Table I** compares the performance as well as pros and cons of the various bone grafts.

TABLE I: BONE GRAFT COMPARISON

Type	Origin	Biologic Mechanism	Advantage	Disadvantage
Autograft	Self	osteoinductive, osteoconductive, osteogenic	excellent success rate, considered the "gold standard", no risk of disease transmission, histocompatibility	limited availability, donor site morbidity
Allograft	Donor	osteoinductive (DBM) and/or osteoconductive	availability	immune response, disease transmission, variable quality/properties
Synthetic Graft	Polycrystalline Ceramic	osteoconductive	availability	immune response, poor tensile strength, graft may resorb too fast or too slow to be effective

An autograft is considered the "gold standard" in terms of performance because it is comprised of all three graft properties (**Table I**). However, the relative proportion of each property depends on whether the bone is cortical or cancellous and its source. For example, autograft cortical bone is less osteoinductive than cancellous bone and is mainly osteoconductive and osteogenic (23, 29). However, allograft bone, cortical or cancellous, can not be osteogenic because it contains no living cells. Allograft bone is osteoconductive only, while demineralized allograft bone is both osteoconductive and osteoinductive. They come in many forms for various uses such as morselized bone to fill voids, or cortical struts for structural applications (29). Synthetic grafts, such as calcium phosphates and calcium sulfate (Plaster of Paris), can be classified as polycrystalline ceramics and are used as

osteoconductive matrices. These calcium phosphate ceramics are available as porous or nonporous blocks of various sizes or as porous granules, they are also available as self-setting pastes whereby a liquid and powder component are mixed together, applied, then set into a hard cement (29). Calcium phosphate-based grafts sometimes also are in the form of synthetic hydroxyapatite. Calicum sulfate (Plaster of Paris) grafts are also used clinically (28). One of the problems with the synthetic ceramic grafts is that they may resorb too quickly or too slowly to be effective. The ultimate goal of these synthetic osteoconductive grafts is to ultimately be replaced by bone tissue. If the synthetic graft resorbs faster than it is being replaced by bone, then a bone vacancy may remain long-term. Alternatively, if the synthetic graft resorbs too slowly, or not at all, it may never be entirely replaced by natural bone. One reason that there are so many type of synthetic ceramic bone grafts available is that clinical needs vary so having a family of such grafts with a wide range of characteristics permits the surgeon to taylor the graft to the specific needs of the individual patient.

5.1 Demineralized Bone Matrix

As mentioned earlier, bone matrix, which is "masked" by the mineral phase of native bone, contains proteins such as BMPs. Demineralization exposes the type I organic bone matrix and therefore, "unmasks" BMPs within the matrix, and increases their bioavailability for interaction with MSCs, making DBM grafts highly osteoinductive as well as osteoconductive by providing a scaffold for new bone to grow on (23). The sequence of events which follows the implantation of DBM graft in vivo is well described; and the induction of new bone is due to the BMP within the matrix (30). Immediately following implantation of DBM into orthotopic or even heterotopic sites, chemotaxis and mitosis of MSCs begins and the remaining sequential bone morphogenesis, in response to DBM,

mimics the natural process of bone development and fracture healing. It is important to mention that when mineralized bone is implanted in a heterotopic site, new bone does not form; however, DBM does produce new bone when used in this fashion, showing how potent it is for producing bone (13).

DBM is prepared by a standardized process in which allogenic bone is crushed to a consistent particle size (typically ranging anywhere from 125-710 μm) followed by demineralization (typically in 0.5N HCl for three hours) (11). The acid can be eliminated by rinsing in sterile water, ethanol and ethyl ether. Although the same basic procedure is followed, variation in particle size, demineralization time, and acid concentrations have been patented by companies to claim advantages and superiority over other products.

DBM is available as a freeze-dried powder, as crushed granules or chips, and as a gel or paste made with a carrier. A carrier may be used due to the fact that DBM can be difficult to handle in the operating room because of the lack of cohesion among the small particles. To overcome handling limitations and to extend DBM grafts to fill larger voids, DBM is offered in combination with a carrier to give the graft the consistency of a paste or putty. Carriers can be of either synthetic or biologic origin. Some examples of carriers used commercially in DBM products are glycerol, hyaluronic acid, collagen, synthetic polymers, and autologous blood (11).

Clinically, DBM has been used for a wide range of orthopedic procedures. DBM has induced bone formation in bone cysts and cavities, application of DBM to long bone non-

unions and acute bone defects from fractures have resulted in successful healing similar to autografts, and DBM can also be used to enhance healing of arthrodesis (fusion of adjacent bones) in the spine and elsewhere (29).

Because DBM is a type of allograft, advantages and disadvantes of DBM are similar. Once again, availability of DBM graft is a significant advantage, however, there is a risk of disease transmission. However, the risk is very low because the DBM donor grafts go through an intense screening process. One large tissue bank that processes DBM reported that no infectious disease transmission occurred from more than 20,000 donors (29).

6. Methods for Measuring Osteoinductivity in DBM

The following in-vitro and in-vivo bioassays have been established for the evaluation of DBM osteoinductivity (11,17):

- In-vitro quantitative BMP ELISA: involves quantitative measurement of BMPs (which are isolated from the DBM using protein extractants such as 4M GuHCl) using a sandwich enzyme-linked immunosorbent assay (ELISA). Initially, antibodies are fixed to a solid surface such as a microplate well surface; the protein extracts are then added to the well and any antigen, or BMP in this case, present binds to the immobilized antibody. After washing away any unbound substances, an antibody-enzyme conjugate specific for a certain BMP is added to the wells and the antibody part of the conjugate binds to the BMP molecules that were bound previously, creating an antibody-antigen-antibody "sandwich". After washing away any unbound conjugate, a substrate is added to the wells to provide color intensity proportional to the amount of BMP bound to the initial immobilized antibody and the reaction is stopped after a set interval. The color intensity

(optical density) of the test specimens at a particular wavelength is then measured with a spectrophotometer and compared with that of a dilution series of standards. It has been found that there is a strong correlation between the BMP content of DBM and the in vivo production of new bone in animal models, providing an important means of screening potential human donors [34].

- In vitro alkaline phosphatase activity assay: assay makes use of the observation that increased ALP activity is found in areas of greater osteogenesis, a result of higher osteoblast activity.   DBM is incubated with mammalian cells under cell culture conditions; non-demineralized bone is used as a control.

- In vivo remineralization assay: rehydrated DBM is implanted into subdermal or muscle pouches of athymic rats or mice with the objective of stimulating new bone formation in the implant.   Athymic rats or mice are used to eliminate the cross-species immune response that would otherwise result when placing human DBM in these animal models. The sample is removed after 28 to 35 days after implantation. The calcium content of the extracted sample is compared to the original implant material.

- In vivo histology assay: Once the DBM is implanted as above, the samples are fixed for histological evaluation of a cross-section of the implanted area; numerical values indicate the amount of new bone formed.

- In vivo micro-CT analysis: micro-CT analysis is utilized to assess quantification of the mineral content, and for 3-D imaging of the entire sample.

B. RELATED LITERATURE

1. BMP Association and Diffusion with respect to DBM

BMPs are bound to the extracellular matrix components such as collagens I and IV, heparin sulphate, heparin, and the bone mineral hydroxyapatite (17). A study relative to BMP association with the bone matrix was performed in which hydrated and anhydrous DBM was incubated at temperatures of 21-65°C for 5 weeks; the osteoinductivity of both samples was examined and the osteoinductivity of the hydrated DBM was much less stable than the anhydrous DBM (31). The authors stated that the collagen/growth factor association protects the structural integrity of the growth factors and when disrupted through conformational changes in the collagen due to heat and water exposure, growth factor activity diminishes. The authors proposed that BMP association with DBM includes a free, loosely bound, and tightly bound state.

Landesman and Reddi suggested a soluble factor from DBM was associated with osteoinduction; after subdermal implantation of DBM in a rat, changes in the osteoinductive potential of DBM during the initial stages of endochondral bone formation was examined (32). Diffusion chambers, constructed with filters of known pore size, permitting or excluding cells from entering the chambers, and containing DBM were implanted into the rats for specific time periods (1–7 days). The chambers were recovered and the osteoinductive potential of the matrix from these chambers was then tested by subdermal re-implantation and assaying the resulting day 11 tissue for ALP activity, and histologically for evidence of chondrogenesis and osteogenesis. It was concluded that after an initial in vivo latent period, the osteoinductive potential of DBM has a half-life of 5–7 days. In another study, the change in measurable BMP-7 concentration in bovine bone during prolonged acid demineralization of up to 24 hours was examined, and the release half-life was found to be

26 hours in the acid medium (33). However, their study was not designed to examine BMP release from DBM in a physiological-like environment.

1. **BMP-7 Release Kinetics**

Recently, the release kinetics of BMP-7 from bovine DBM in an in vitro physiological buffer solution as a function of DBM particle size was examined by Pietrzak et. al. (27). One-and-one-half grams of DBM with three particle size ranges (<106, 106-300, and 300-710 μm) derived from bovine cortical bone was extracted in 40 ml of Sorenson's buffer at room temperature for specific time periods up to 7 days. The BMP-7 concentration of the DBM, obtained following GuHCl extraction of the DBM to remove remaining BMP-7, and buffer was measured at each time point using the ELISA assay. It should be noted that it is commonly assumed that guanidine HCl quantitatively extracts, or removes, all BMPs from DBM to allow them to be assayed. Guanidine HCl is a dissociative extraction method - it denatures BMPs which changes their association with DBM, causing it to be released. After the denatured BMPs are released, they must be renatured to their native conformation by removing the guanidine, typically performed by dialysis. Based on measurement of the concentration of BMP-7 in the buffer, the elution of BMP-7 for all three particle sizes from the DBM was rapid for the first 8 hours, then slowed down for the remainder of the study, but showed no sign of elution nearing completion by 7 days (see **Figure 2**).
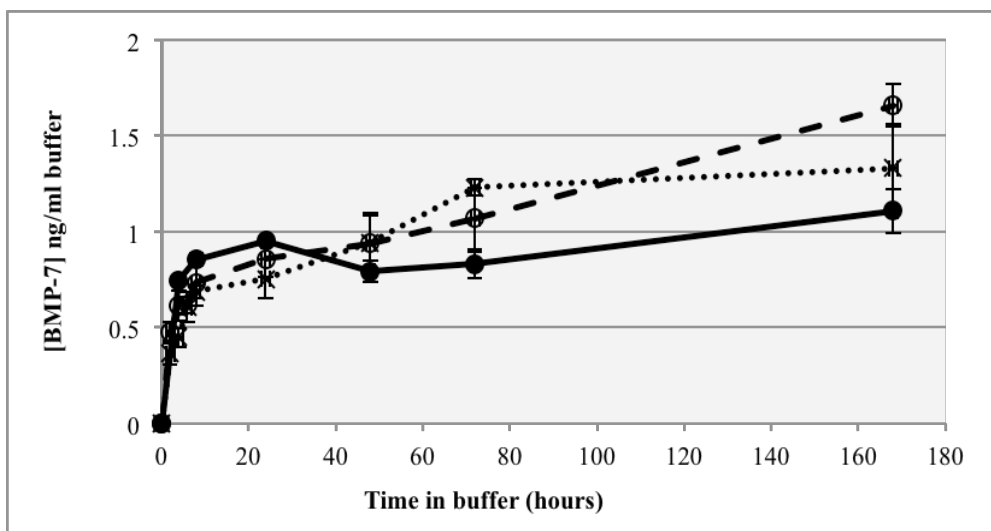
Figure 2. Measured BMP-7 concentration in the Sorensen's Buffer as a function of time and particle size: small (<106 μm) solid line, medium (106-300 μm) dashed line, and large (300-710 μm) dotted line (mean ± SD), Pietrzak, et al (27)

Measurement of the residual BMP-7 remaining in the DBM over time yielded unexpected results; after the BMP-7 content declined for the first 4-6 hours, it continuously increased for the remaining amount of time for all three particle sizes (**Figure 3**).
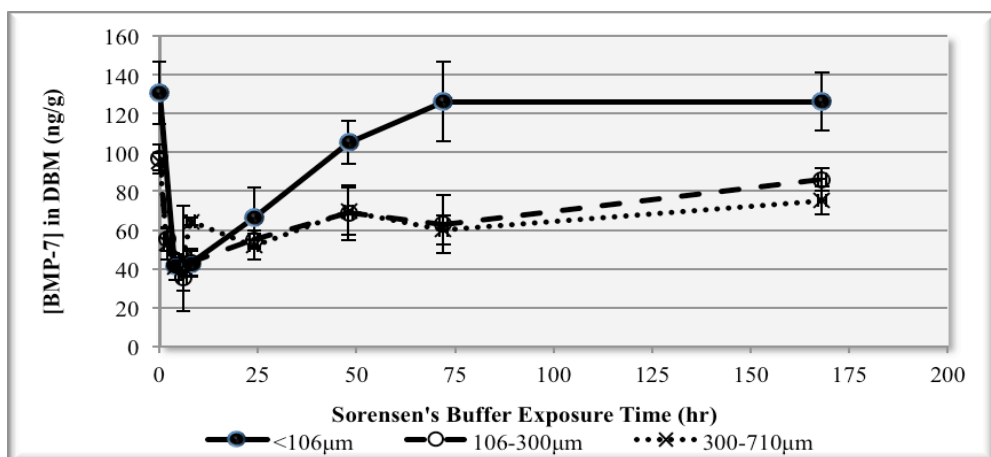
Figure 3. [BMP-7] in DBM for various particle sizes (27)

To explain the time profile of BMP-7 in the bone matrix, it was suggested that BMP-7 is associated within two compartments in DBM, differing by how the BMP-7 was associated with the matrix; "tightly" or "loosely" bound to the matrix. The rapid increase of BMP-7 in the buffer and initial decline of BMP-7 in the DBM was hypothesized to be due to the rapid release of BMP from the loosely bound compartment to the surroundings. It was proposed that the slower, prolonged increase of BMP-7 in the buffer and its unexpected concomitant increase in the DBM was due to the movement of BMP-7 between these two compartments, i.e., from the tight to the loose compartment. In other words, it was proposed that the tight compartment acted as a BMP reservoir while the loose compartment was the conduit through which the BMPs passed to the surrounding medium. Another factor they considered was the possibility that guanidine extraction may not remove all of the BMPs from DBM as is commonly assumed. They suggested this through mass balance calculations which showed that there was significantly more BMP-7 in the closed DBM/buffer systems after 7 days than there was initially. If the guanidine extraction removed 100% of the BMP-7 from the DBM at each time point so that it could be assayed, along with that which was in the buffer compartment, then the total BMP-7 in the closed system should have been constant over the 7-day incubation. As such, they suggested that the efficiency with which guanidine removes BMPs from DBM may be time-dependent in that it is a function of the hydration history of the DBM and may only remove BMPs from the loose compartment.

Pietrzak's study had some limitations (27). The maximum exposure to SB was only 7 days, at which there was no indication that the elution was reaching completion; therefore, long-term elution characteristics could not be characterized. Secondly, only elution of BMP-

7 was measured, although other significant members of the BMP family are present in the DBM, including BMP-2 and BMP-4. Also, only one type of protein extraction procedure, GuHCl, was used to extract BMP-7 out of DBM when other procedures exist. As stated above, GuHCl is a protein denaturant and functions by changing the conformation of BMPs, thereby altering their native association with bone matrix, causing their release; whereas another type of extraction method, such as collagenase digestion, enzymatically breaks down bone matrix which releases BMPs. It is possible various extraction procedures yield different results.

C. **PURPOSE AND AIM OF STUDY**

Although DBM grafts have been used for decades, there is a limited amount of literature to be found in regards to the DBM BMP release kinetics of BMPs (17, 27 31-33).

Such knowledge is important to better understand bone biology, the nature of osteoinduction, and can help in the design of improved DBM grafts.

The aim of this study was to extend the work of Pietrzak et. al. (27) to better understand the characteristics and elution rates of BMP-7 out of bovine DBM (in-vitro) for a longer period of time, using various protein extraction procedures as a probe of the association of BMP-7 with the DBM. For clinical significance, the release kinetics of BMPs-2, -4 and -7 from a human DBM product currently being used in surgical procedures (Bonus II DBM, Biomet, Inc., Warsaw, IN) was also examined.

D. **HYPOTHESIS**

Because the majority of bone development in the bone healing process is complete by 12 weeks (11), it is likely BMPs have completed their role by then. Therefore, it was hypothesized that the BMPs would completely elute out of DBM within 12 weeks.

Pietrzak, et al (27), concluded that the guanidine extraction method, commonly believed to quantitatively extract all BMPs from DBM for measurement, does not remove all of the BMPs from DBM, but rather, appears to differ in its ability to extract BMPs as a function of the duration of DBM buffer exposure. Because collagenase digestion enzymatically breaks down bone matrix to release BMPs, it was hypothesized that the ability of this method to extract BMPs from DBM would be independent of buffer exposure time. By using collagenase digestion in this study, it was predicted that the elution profile of the BMPs in DBM would display a simple exponential decrease over-time, rather than decrease and then increase, as in Pietrzak, et al (27). Finally, the elution profiles of BMPs-2, -4, and-7 were hypothesized to be similar to each other due to their similar characteristics.

E. SIGNIFICANCE OF STUDY

Below is a detailed list of how a better understanding of the release kinetics of BMPs out of DBM may be beneficial:

1. DBM grafts are often comprised of particulate DBM combined with a carrier to improve the intraoperative handling properties. It is possible that the carrier can impede, or otherwise alter, the release of BMPs from DBM, which may have clinical consequences. Knowledge of the elution profiles or rate of release of BMPs-2, 4 and 7 (due to their critical role in bone formation) is important for preparation and design of an optimal

DBM graft. The characteristics of an optimal DBM graft carrier reside in how it affects the release of osteogenic factors such as BMPs from the DBM. Better bone forming results have been obtained using slower dissolving carriers because they assist in the prolonged availability of the DBM, and therefore osteogenic factors, at the site of implantation (26). Alterations of DBM graft may include amount of demineralization, particle size, method of sterilization, the concentration of actual DBM in the graft product, and the nature of the carrier (11).

2. Companies such as Medtronic and Stryker clinically utilize rhBMP-2 and rhBMP-7, respectively, for example. The rhBMP is initially freeze-dried, then reconstituted with sterile water in the operating room and applied to a collagen sponge by soaking it in the aqueous solution containing the rhBMPs for at least 15 minutes, followed by being appropriately cut and placed at the site of the bone defect. Based on studies done on rhBMPs, the genetically engineered BMPs are added to the sponge in quantities that are approximately a million times higher than the amount in the actual DBM to induce adequate bone formation (16). For example, the BMP-2 concentration in a DBM graft is approximately 20 ng/g (34) whereas the concentration of rhBMP-2 clinically used is typically mg per unit volume of matrix (35). The long-term effect of such high concentrations of rhBMPs is unknown. Knowledge of the release kinetics of BMPs from DBM may provide a good target to be matched by these recombinant products. This may lower the required amount of rhBMPs, reducing cost and increasing safety.

3. There is still much unknown about the bone healing cascade and, to the extent that BMPs play an integral role in this cascade, characterization of the release kinetics may help

increase the understanding of the bone healing process and help identify other possible clinical interventions.

4. Knowledge of the distribution of BMPs in bone would help further the understanding of basic bone biology.

5. Optimization of the method of extracting residual BMPs from DBM can be useful for more reliably measuring the BMP content of DBM as a better means to screen potential donors of bone tissue.

II.   MATERIALS AND METHODS

A. DBM SOURCE MATERIAL, CHEMICALS, AND EQUIPMENT,

Bovine long bone, from which the particulate bovine DBM was derived, was obtained from Biomet, Inc., Warsaw, IN.   Human DBM was obtained from Biomet Biologics (Warsaw, IN) in the form of a clinical product, i.e., Bonus II DBM (cat. No. 48-DBM10). All of the Bonus II DBM product was of the same lot number, signifying that it was all derived from the same human donor.

The following chemicals were used in this study and were reagent grade unless otherwise specified, 1) sodium phosphate dibasic anhydrous (Na_2HPO_4), p/n s93376, Fisher Scientific, Fair Lawn, NJ; 2) potassium phosphate monobasic anhydrous (KH_2PO_4), p/n 191430, MP Biomedicals, Solon, OH; 3) sodium azide (NaN_3) S2002, Sigma-Aldrich, St. Louis, MO; 4) benzamidine-HCl, p/n 199001, Calbiochem, Japan; 5) 6-amino hexanoic acid, p/n A14719, Alfa Aesar, Heysham, Lancashire; 6) 4-(2-aminoethyl) benzenesulfonyl fluoride HCl, p/n 50-121-6203, Fisher Scientific, Fair Lawn, NJ; 7) 1M HCl, p/n NC-1193, The Science Company, Denver, CO; 8) Tris(hydroxymethyl)aminomethane-HCl (Tris), p/n H5131, Promega, Madison, WI); 9) GuHCl , p/n 194826, MP Biomedicals, Solon, OH; 10) collagenase, p/n LS005275, Worthington, Lakewood, NJ; 10) 36-38% (11.6M) HCl, cat/ no. NC-1193, The Science Company, Denver, CO. Deionized water was used to prepare all solutions.

The ELISA kits, used to measure the BMP-2, -4, and -7 concentrations, were Duoset DY354, Quantikine DBP200, Quantikine DBP400, and Quantikine DBP700, they were obtained from R&D Systems, Minneapolis, MN.

Major equipment items included 1) MF 10 Basic bone mill (IKA-Werke GMBH and Co., Staufen, Germany), 2) a stainless steel industrial food disposal unit, model SS-75-27, Emerson Electric, Racine, WI; 3) automatic mechanical sieve shaker, RX-29 Rotap, W.S. Tyler, Mentor, OH; 4) stainless steel sieves with pore sizes of 25, 106, 250, 300, 425, 500, 600, and 710 μm, ASTM E-11 specification, VWR, West Chester, PA; 5) Advantage lyophilizer, VirTis/SP Industries, Warminster, PA; 6) 50ml polypropylene conically-tipped tubes with screw caps, p/n 10-9502, Biologix, Shandong, Chin, 6) 15ml polypropylene conically-tipped tube with a screw cap, p/n 10-9152, Biologix, Shandong, China; 7) revolving plate, KJ-201BD Oscillator, Wincom Company, LTD., Hunan, China; 8) centrifuge, Centra CL2, International Equipment Company, Boston, MA; 9) cellulose dialysis tubing with molecular weight cutoff of approximately 12,000D, 25 mm flat width, p/n D9777, Sigma-Aldrich, St. Louis, MO; 10) incubator, model 1530, VWR Scientific, Radnor, PA); 11) SpectraMax Plus spectrophotometer, Molecular Devices, Sunnyvale, CA.

B. METHODS

1. Bovine DBM Study Preparation

1.1 Preparation and Characterization of Bovine DBM

      1.1.1   Bovine Bone Processing

Due to similarities to human cortical bone (33) (**Table II**), bovine cortical long bone was used as a model system for convenience to avoid the cautious handling and limited availability of human tissue.

TABLE II: COMPOSITIONAL COMPARISON OF BOVINE AND HUMAN CORTICAL BONE (33)

Component	Bovine Cortical Bone	Human Cortical Bone (Average ± SD)
Hydroxyapatite (wt%)	68.8	67.7 ± 1.3
Calcium (wt%)	25.7	25.5 ± 0.9
Lipid (wt%)	0.1	1.1 ± 1.5
Matrix (wt%)	31.1	31.9 ± 1.1
Calcium/Hydroxyapatite (w/w)	0.374	0.380 ± 0.015
Hydroxyapatite/Matrix (w/w)	2.21	2.10 ± 0.09

DBM was prepared using methods previously described (10, 23-24). Soft tissue from fresh bovine cortical long bone was removed, the bone was cut into cortical rings approximately 6mm wide, then quartered. Bone chips were created by processing the segments in water with a stainless steel industrial food disposal unit. The chips were then mechanically shaken in warm water for ten minutes with a commercial mechanical paint

shaker/mixer to remove any residual, adherent soft tissue. The water extract/tissue debris was discarded and the cleansed chips were lyophilized and milled to particulate form.

1.1.2   Bovine Bone Particle Size Distribution

The particle size distribution (106-710 μm) of the bone powder was determined by stacking sieves, in order of bottom to top, of 106, 250, 300, 425, 500, 600, and 710 μm mesh, placing the bone powder on the top sieve, and using an automatic mechanical shaker for 15 minutes. This allowed the bone powder to be size-stratified on the various sieves. The size fractions were then weighed and uniformly recombined for a single, overall, particle size range of 106-710 μm.

1.1.3   Demineralization of Bovine Bone

Sorenson's buffer (SB) was made in 12 L batches as follows. Twelve liters of deionized water, 123.6 g of Na_2HPO_4, and 32.28 g of KH_2PO_4 were placed in a 15 L vessel. The contents were magnetically stirred for one hour to yield pH ~7.3 buffer.

A total of 1400 g of lyophilized bovine bone powder was demineralized. The bone particles were demineralized at 24° C, in a 19 L polycarbonate vessel containing a magnetically stirred bath of 0.5N HCl, with 50 ml of 0.5N HCl per gram of bone powder. The vessel was covered with Parafilm and first mixed by inversion. The vessel was then set on a magnetic stir plate for 60 minutes, adjusting the rotational speed to create a small vortex. After 60 minutes of demineralization, the vessel was removed from the stir plate, and the bath with the DBM was poured through a stainless steel sieve with a pore size of 25 μm to collect the DBM. Once the DBM was collected, it was washed and neutralized

by pouring approximately 3 L of deionized water and 3 L of SB through the DBM along with manual agitation for approximately one minute, each, to remove the residual acid until the pH of the DBM, as tested with pH paper, became neutral.

After the wash was complete, the wet DBM was pressed against the mesh to further drain the wash water/buffer. The DBM was then placed on a stack of filter papers and rolled and pressed to further express excess water. The DBM was then placed in freeze-drying bags, sealed, and flattened by pressing on the bag to create a large surface area to facilitate freeze-drying. The freeze-drying bag with the DBM was placed in a freezer below -20° C to freeze the DBM. Note that although sealed, the bags were still permeable to moisture to allow freeze-drying to occur. The bag was then transferred to the lyophilizer where a 48 hour cycle was used to freeze dry the DBM.

1.1.4   Calcium and Water Content Analysis of Bovine DBM

An aliquot of the freeze-dried DBM was sent to Sherry Laboratories, Inc. (Daleville, IN) for analysis of residual calcium to determine the extent of demineralization. The method used was to digest the organic matter and measure the calcium content using atomic absorption spectroscopy with the appropriate standards. The water content of the freeze-dried DBM was measured gravimetrically by weighing an aliquot before and after heating at 105° C for 3 hours.

The water content was calculated as shown in **Equation 1**:

$$\% \ H_2O = (\text{mass undried DBM} - \text{mass dry DBM}) / \text{mass undried DBM} * 100\% \qquad (1)$$

1.2 <u>Experimental matrix for Bovine DBM</u>

There were several segments of the experimental protocol that were selectively varied.
These were 1) exposing the DBM to SB for varying lengths of time for BMP-7 elution, 2) the
method of extracting residual BMP-7 from the DBM following SB exposure for BMP-7
measurement (termed "analytical extraction), and 3) whether protease inhibitors (PI) were used.
The experimental matrix is shown in **Table III** with the details provided in the following
sections.   For each set of experimental conditions, at least two independent SB runs were
performed.

TABLE III: EXPERIMENTAL MATRIX FOR BOVINE

Variable	Experiment 1 (GuHCl+PI)	Experiment 2 (GuHCl)	Experiment 3 (CD)
Duration of SB exposure	0, 2, 4, 6, 8, 24 hr, 2, 3, 7, 14, 21, 28 days	0, 2, 4, 6, 8, 24 hr, 2, 3, 7, 14, 21, 28, 42, 56, 84 days	0, 2, 4, 6, 8, 24 hr, 2, 3, 7, 14, 21, 28, 42, 56, 84 days
Protease Inhibitors	Yes	No	No
Analytical extraction method	GuHCl	GuHCl	Collagenase Digestion

* CD = collagenase digestion

1.2.1   <u>Elution of BMP-7 from Bovine DBM into SB</u>

50ml polypropylene conically-tipped tubes with screw caps were used as the
elution vessel. DBM (1.5 g) and 40 ml of SB, with 0.05% sodium azide, to prevent
bacterial contamination and growth (36-38), were placed in the elution vessel and capped.

The capped elution vessel was then secured horizontally on an automated revolving plate shaker that was set to revolve at a speed of 80 rpm in the horizontal plane to continuously mix the contents at room temperature (25-27° C).

The procedure of Pietrzak, et al (27) was followed to process the SB and DBM after exposure to SB for the prescribed time interval. After the respective time interval was reached (ranging from 2 hours to 84 days, see **Table III**), the elution vessel was removed from the revolving plate and placed stationary in a vertical position for 5 minutes to allow the DBM to settle. The SB containing the eluted BMP-7 was pipetted into a similar vessel and frozen at < -20° C for later BMP-7 analysis. The DBM pellet was washed to remove residual BMP-7 unassociated with DBM by adding 40 ml of fresh SB to the elution vessel and resuspending the pellet by inversion, then allowing it to settle for 5 minutes once more. The wash SB was discarded and the washed DBM pellet was placed on several layers of filter paper to drain excess SB, and freeze-dried.

Proteolytic enzymes are present in bone matrix and may contribute to the degradation of BMPs (39-40) but it was unclear whether this would affect the results of this study. Consequently, one set of experiments included the use of protease inhibitors (PI) in the SB (See **Table III**). The cocktail of PIs used in the SB included 5mM benzamidine-HCl, 0.1M 6-amino hexanoic acid, and 0.5mM 4-(2-aminoethyl) benzenesulfonyl fluoride HCl. These PIs are commonly used to prevent the possible enzymatic degradation of BMPs (25,41-42), and such a combination of PI has also been previously used (42). Benzamidine-HCl and 4-(2-aminoethyl) benzenesulfonyl fluoride

HCl are serine protease inhibitors, while 6-amino hexanoic acid is a lysine protease inhibitor, which inactivate a variety of proteases that bind to the respective amino acid residues. These proteases include chymotrypsin, urokinase plasminogen activator, kallikrein, plasmin, thrombin, furin, and trypsin (43).

1.2.2   Analytical Extraction of BMP-7 from Bovine DBM

As BMP-7 elutes from DBM into SB, at any given time some of the BMP-7 will have moved from the DBM into SB with the balance remaining associated with the DBM. To measure the amount of BMP-7 remaining in the lyophilized DBM following SB elution, a stronger extraction medium (analytical extraction) than SB must be employed with the goal of quantitatively removing all of the remaining BMP-7 from the DBM.

Two different analytical extraction methods, dissociative and non-dissociative, were employed to extract BMP-7 from the SB-eluted, freeze-dried DBM for quantification by ELISA. Guanidine hydrochloride (GuHCl), used for dissociative protein extraction, denatures BMPs which allows their release from the DBM and is a commonly used extractant (23, 25, 27, 33-34, 40-41). However, the released denatured BMPs must be renatured to their native conformation to allow their measurement by ELISA. Renaturation spontaneously occurs once the GuHCl is removed, such as can be done via dialysis (34) prior to measurement.

Collagenase digestion, the non-dissociative BMP extraction method, does not directly affect the BMPs, but destroys the substrate to which the BMPs are associated

with, allowing their release (34). This method of BMP extraction has also been used in previous studies (42,44).

1.2.2.1 BMP Extraction of Bovine DBM Using GuHCl

Approximately 0.3 g of lyophilized DBM from each SB-exposure time interval was weighed and added into a 15 ml polypropylene conically-tipped tube with a screw cap.

Tris(hydroxymethyl)aminomethane-HCl (Tris) buffer was made by adding 1M HCl drop-wise to 0.05M Tris while magnetically stirring to reduce the pH from ~10.4 to ~7.3. GuHCl was then added to this stock solution to produce 4M GuHCl/0.05M Tris-HCl.

The guanidine extraction was performed per Pietrzak, et al (27). Using an autopipette, 5 ml of 4M GuHCl/0.05M Tris-HCl was added into each tube containing the DBM. The tubes were capped and placed horizontally on the automated revolving plate set to revolve at 80 rpm in the horizontal plane, permitting continuous agitation for 24 hours at 4° C. The tubes were then centrifuged at 2,000 rpm for 5 minutes and approximately 3.5 ml of supernatant (GuHCl extract) was pipetted into another 15 ml capped tube and stored at 4° C. Approximately 1.5 ml, or so, of the GuHCl extract was unavailable as supernatant as it had absorbed into the DBM particles as well as resided in the space between DBM particles in the pellet. The DBM pellet in the original tube was left intact and 5 ml of fresh 4M GuHCl/0.05M Tris-HCl was added into it for a secondary extraction, this time for 4 hours. Following centrifugation, approximately 3.5 ml supernatant was similarly obtained and combined with the first 3.5 ml aliquot for a total of approximately 7 ml. The fully extracted DBM was then discarded.

Dialysis of the GuHCl extract was performed to remove guanidine and allow renaturation of the BMPs. Specifically, the 7 ml of GuHCl extract was placed in a dialysis tube with a molecular weight cutoff of approximately 12 kDa and dialyzed against 350 ml of 0.05M Tris-HCl (pH~7.3) at 4° C for 15 hours to remove the GuHCl (**Figure 4**).
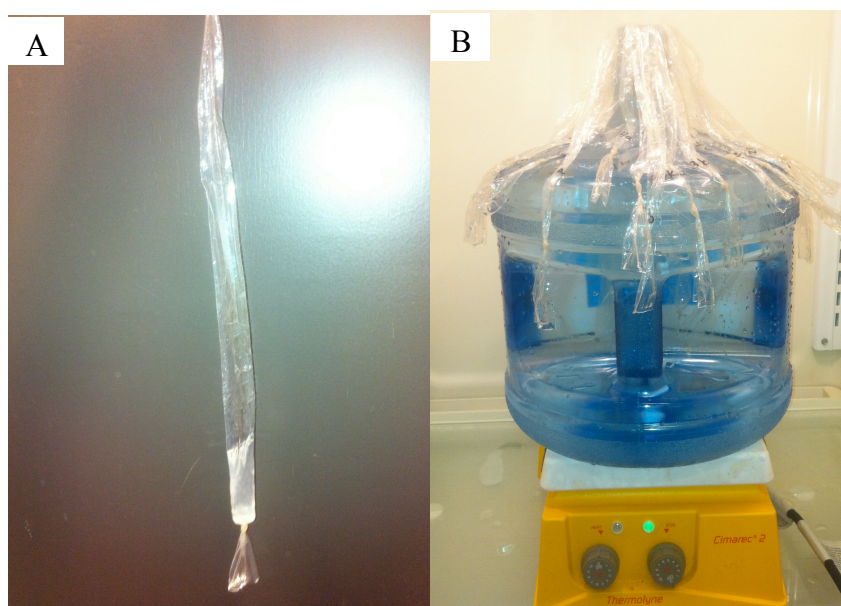
Figure 4. A. A single dialysis tube containing approximately 7 ml of GuHCl extract. **B.** Several labeled dialysis tubes, each containing 7 ml of GuHCl extract from separate experiments, dialyzed together in an 11.4 L polycarbonate vessel, while maintaining the appropriate overall ratio of buffer to GuHCl extract volume (50 ml 0.05M Tris-HCl/ml GuHCl extract).

Multiple specimens were dialyzed simultaneously in the same reservoir of 0.05N Tris-HCl buffer while maintaining an overall ratio of 50 ml of buffer per ml of GuHCl extract. The 0.05M Tris-HCl was then replaced with fresh buffer and the dialysis was continued for another 10 hours at the same temperature. Dialysis did not effect the volume of extract contained in the tubing. The dialyzed extract was then transferred from the dialysis tubing into a 15 ml polypropylene tube with a screw cap and frozen below -20° C for later BMP-7 analysis. Note that the GuHCl extraction of BMP-7 from DBM for Experiment 1 (see Table III) differed from that performed for Experiment 2 in that the same PI cocktail described above was included to prevent BMP-7 degradation. Therefore, 4M GuHCl/0.05M Tris-HCl/PI was used for the analytic extraction for Experiment 1 (**Table III**).

1.2.2.2 BMP Extraction of Bovine DBM Using Collagenase Digestion

Collagenase was used for the nondissociative extraction of BMP from DBM. DBM (1 g) was mixed with 6.5 ml of 0.2M Tris-HCl (pH~7.3) containing 20 collagenase degrading units (CDU)/ml in 15 ml conical tubes. The tubes were placed on a revolving plate moving at 80 rpm for 17 hours at 37° C in an incubator (1530, VWR Scientific). The specimens were then removed and centrifuged at 2000 rpm for 20 minutes. The supernatant was collected and frozen below -20° C for later BMP-7 analysis (44).

2. **Human DBM studies**

As described above, most of the experiments performed in this study investigated the elution of BMP-7 from bovine DBM. In an attempt to generalize the study beyond this

model, the elution of three BMPs, i.e., BMP-2, BMP-4, and BMP-7 from human DBM were also investigated. In particular, a clinically available human DBM product (Bonus II DBM) was used for this phase of the study.

2.1 Bonus II DBM Characteristics

Bonus II DBM (Biomet Biologics, Warsaw, IN) is a product currently marketed for clinical use. It is comprised of particulate human DBM that has been combined with a carrier derived from human DBM from the same donor to improve handling properties. During manufacture, a small amount of particulate DBM is mixed with saline, then exposed to a thermal cycle to denature the collagen and produce gelatin. Non-thermally processed (active) particulate DBM is then mixed with the carrier and lyophilized. Upon hydration of the product in the operating room with blood, bone marrow aspirate, saline, antibiotic solution, etc., it becomes putty-like in consistency, which allows the surgeon to press it into bone voids or gaps. Only the non-thermally processed DBM contains active BMPs since those present in the carrier DBM are thermally denatured.

2.2 Preparation of Bonus II DBM for study

The graft preparation system (gps) containing the Bonus II DBM consisted of a plastic syringe-type apparatus with a plunger and a side Luer-type port containing a valve (note that when nothing was connected to the port the valve is closed but when a syringe was connected to this port the valve is open), and a distal circular 1.6 cm diameter exit port. As provided, there were two freeze-dried cylindrical DBM "logs" in the gps, each of which had a diameter, length and weight of approximately 1.5 cm, 2.5 cm, and between 1.7-1.8 g, respectively (**Figure 5**).
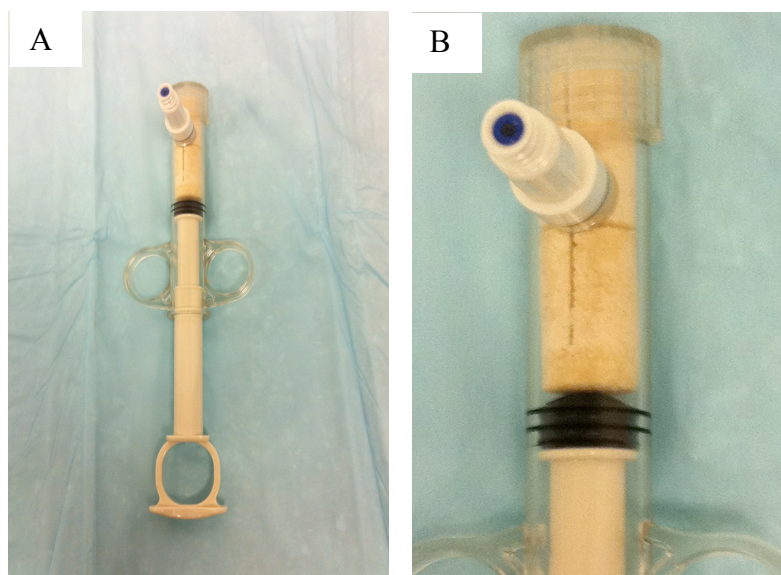
Figure 5. A. Image of gps. B. Close-up of gps, showing the two DBM logs in the syringe; also showing how each log is vertically cut approximately 4/5 down the middle to aid in hydration.

A single log (one of the two supplied in the gps) was sufficient for use in each extraction experiment, hence each gps provided sufficient DBM for two experiments. Once the dry weight of a DBM log was recorded, it was placed back into the gps by itself and hydrated with SB.   Hydration was achieved as follows.

First, an empty 30cc syringe was attached to the gps side port (**Figure 6**).

Figure 6. 30 cc vacuum syringe attached to gps side valve

The plunger of this syringe was then was pulled back as far as it would extend to draw a vacuum in the gps, then the syringe was removed from the side port to close the valve and seal in the gps vacuum. Next, a smaller syringe containing at least 5 ml of SB was attached to the gps side port. In the process, the valve opened and the gps vacuum drew in approximately 5 ml of SB to hydrate the single DBM log. The log was allowed to hydrate for 5 minutes causing it to change from a rigid and hard solid to a soft "putty-like" consistency. The plunger of the gps was then advanced to eject the hydrated DBM log.

2.3 Elution of BMPs-2, 4, and -7 from human DBM into SB

The elution of BMPs from the human DBM into SB was performed similarly to the procedure used for bovine DBM as described above, for exposure intervals of 0, 2, 4, 6, 8, 24, 48, 72 and 168 hours (pH 7.36, 26-27.5° C) with two independent SB extractions performed for each time point. Approximately one hour was required for the hydrated DBM log to fully disperse in the SB while on the revolving plate (**Figure 7**).
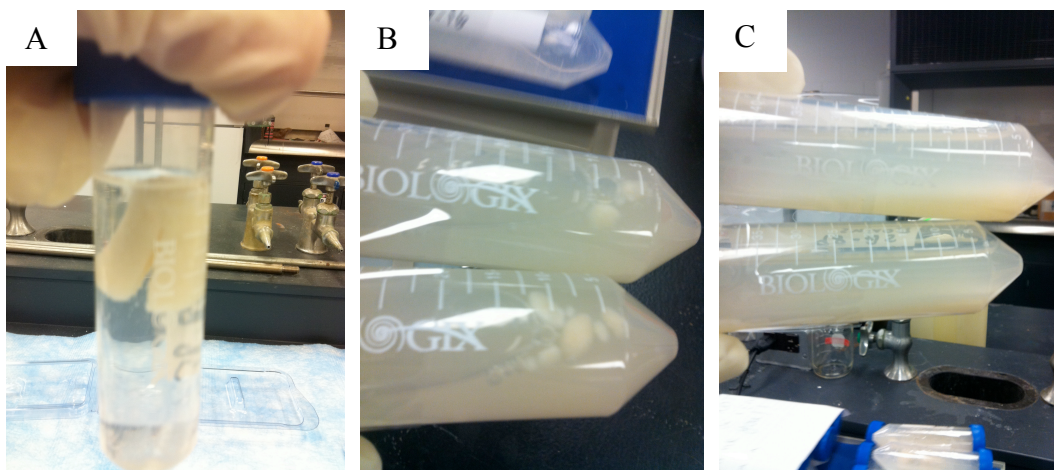
Figure 7. Appearance of Bonus II DBM log (A) just after ejection into SB before placement on the rotating plate, (B) after 30 and, (C) 60 minutes on the rocking plate.

One procedural difference with the human DBM was that since 5 ml of SB was used to hydrate the DBM log in the gps, the hydrated log was placed in an additional 35 ml of SB so that

the total volume of SB in the extraction vessel was 40 ml to equal the amount used in the bovine DBM experiments. Another difference was that unlike the case with the bovine DBM whereby a pellet formed under the action of gravity, 5,000 rpm centrifugation was required to fully separate the DBM pellet from the SB after exposure, perhaps due to leaching of the DBM carrier into the SB causing its viscosity to increase. As was the case with the bovine DBM experiments, the human DBM pellet was lyophilized and the SB supernatant was frozen at <20° C for later analysis.

2.4   BMP Extraction of Bonus II DBM

BMP extraction of the Bonus II DBM was performed using GuHCl, as done for the bovine studies above (excluding PI), and the extracts were frozen at < -20° C for later analysis.

3. Measurement of BMP Content in Bovine/Bonus II DBM and SB

At each time point of exposure of DBM to SB in the extraction vessel, the BMP content of both the lyophilized DBM and the SB was measured.   However, at time zero, corresponding to the initial condition in which the DBM had not yet been mixed with the SB in the extraction vessel, the SB was not assayed since it obviously did not contain any BMPs.

As stated above, the bovine DBM experiments had only BMP-7 measured using the DY-354 Duoset ELISA kit. The human DBM experiments had BMP-2, BMP-4, and BMP-7 measured with the DBP200, DBP400, and DBP700 Quantikine ELISA kits, respectively. The DY-354 kit was more labor intensive than the others as it required the user to first coat the plate wells with BMP-7-specific capture antibody before performing the assay; instructions included

with the DY-354 kit can be found in **Appendix A**. The various Quantikine kits, however, included wells that were pre-coated with the specific capture antibody. It should be noted that although these kits are specific for measurement of their respective human BMPs, bovine BMPs are very similar to their human analogs and a human BMP-7 ELISA assay has been used before to measure bovine BMP-7 (33).

In practice, the frozen analytical extracts of the bovine and human DBM specimens were thawed, as were the frozen SB specimens that were retained from the extraction experiments. The ELISA assays were performed on these thawed specimens per the detailed instructions included with the kits. A brief description of the method follows.

The wells in a 96-well plate were first coated (if they did not come pre-coated) with a monoclonal antibody specific for the BMP to be tested. BMP standards and experimental samples were then pipetted into the wells. Any of the specific BMP present was then bound to the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for that BMP was added to the well. The antibody moiety binds to the immobilized BMP with the enzyme moiety available to react with an appropriate substrate. Thus, an antibody-BMP-antibody "sandwich" is formed. Following a wash to remove any unbound antibody-enzyme complex, the amount of bound enzyme was proportional to the amount of bound specific BMP. A substrate solution was then added to the well with the rate of color development proportional to the amount of bound enzyme. After a fixed time interval of enzyme-substrate reaction (20 to 30 minutes, depending on the kit used), the color reaction was stopped and the color intensity at 450 nm was measured (with wavelength correction at 540 nm

to correct for optical imperfections in the plate) and compared to a standard curve. Typically, two to four ELISA wells were processed for each analytical extract that was obtained. Results were given in pg/ml values.

3.1 Units used to Express BMP Concentration

Since the only source of BMPs present in the experimental extraction vessels was DBM, the amounts measured in both the DBM and the buffer were normalized per gram of lyophilized DBM contained in the vessel, e.g., ng BMP-7/g DBM for the concentration in the DBM or SB. In the case of the latter, it would be the total amount of BMP-7 in the entire 40 ml of SB divided by the amount of DBM contained in the vessel. For all the GuHCl extractions, note that although 3.5 ml of analytical extract was available after each segment of the 2-step analytical extraction process for a total of 7 ml recovered, the ng of BMP per ml of analytical extract was multiplied by 10 ml, since that was the total volume used to obtain the total amount of BMP remaining in the DBM that had been originally placed in the extraction vessel, as displayed in **Equation 2**:

$$[BMP_{DBM}] \text{ (ng BMP/g DBM)} = \frac{[BMP_{GuHCl}](pg/ml)*10(ml)}{m_{DBM,GuHCl}(g)*1000 \text{ (pg/ng)}} \qquad (2)$$

where $[BMP_{DBM}]$ is the concentration of BMP remaining in the DBM specimen at a given time point, $[BMP_{GuHCl}]$ is the BMP concentration of the analytical extract that was added to the respective ELISA well, and $m_{DBM,GuHCl}$ is the mass of DBM used for the analytical extract.

A similar equation was used to measure the concentration of BMP remaining in DBM for the collagenase digestion analytical extraction method, however, the 10 ml was replaced by 6.5 ml (since this was the extract amount of collagenase in Tris-HCl used as mentioned above).

$$[BMP_{SB}] \, (ng \, BMP/g \, DBM) = \frac{[BMP_{ELISA}](pg/ml)*40(ml)}{m_{DBM,vessel} \, (g)*1000 \, (pg/ng)} \qquad (3)$$

[BMP$_{SB}$] is the concentration of BMP in the SB, [BMP$_{ELISA}$] is the BMP concentration of the SB solution added to the respective ELISA well, and m$_{DBM,vessel}$ is the mass of DBM used in the elution vessel containing 40 ml SB.

The normalized BMP concentrations in the DBM and SB were averaged for each respective time interval and run as displayed in **Equations 4** and **5**.

$$[BMP_{DBM-AVG}] \, (ng \, BMP/g \, DBM) = \frac{\Sigma[BMP_{DBM}] \, (ng/g)}{N} \qquad (4)$$

$$[BMP_{SB-AVG}] \, (ng \, BMP/g \, DBM) = \frac{\Sigma[BMP_{SB}] \, (ng/g)}{N} \qquad (5)$$

N is the number of wells used for each time point. N was typically 4 to 8 unless procedural difficulties were encountered which resulted in a smaller number of replicates.

4. Statistical Analysis

Statistical comparison of multiple means was made using a one-way analysis of variance followed by a *post hoc* Student-Newman-Keuls test with $p < 0.05$ considered significant. Comparison of two means was performed with a Student t-test. Segments of the various BMP time profiles were linearized by the method of least squares linear regression.

III.   RESULTS AND DISCUSSION

A. RESULTS

1. Characterization of Bovine DBM

1.1 Particle Size Distribution

The bone particle size distribution used for the preparation of bovine DBM is displayed in **Table IV.**

TABLE IV: BOVINE BONE PARTICLE SIZE DISTRIBUTION

Particle Size Range (µm)	Weight %
106-250	35.8
250-300	11.1
300-425	19.9
425-500	13.1
500-600	10.9
600-710	9.2

1.2 Calcium and Water Content Analysis of Bovine DBM

To verify complete demineralization (45), the residual calcium in the lyophilized DBM was measured to be <0.1 wt%, compared to 25.7 wt% for undemineralized bovine bone (33).

To assure the water content of the DBM met the specification of the AATB for lyophilized tissue (<6wt%) (46), the water content was calculated to be 0.7wt%. Bovine Results and Analysis

Weight of the bovine DBM used, and raw data obtained from ELISA readings can be found in **Appendices B-D**. There were three independent runs using GuHCl only as the analytical extract (no PI), see **Appendix C**, with two runs having the longest DBM/SB extract at 28 days and the third up to 84 days. The initial concentrations of BMP-7 in the DBM in one of the 28 day runs and the 84 day run were 25.5±1.7 and 24.2±5 ng/g, respectively. The initial concentration in the second 28 day run was 75.4±19.1 ng/g, or three times that of the others. This was significantly different that the other two initial concentrations The analytical procedure for that anomalous run was re-examined and the calculations were re-checked, but to no avail. The technical support staff of the manufacturer of the ELISA assay was also consulted, but this yielded no explanation. As such, the run with the spuriously high initial BMP-7 concentration was considered to be an outlier and not included in the analysis. It should be noted, however, that despite the high initial DBM BMP-7 concentration, that run did exhibit the same time course or profile of BMP-7 concentration in the DBM and in the SB as the other two runs. The results of the two runs with equivalent initial concentrations of DBM BMP-7 were combined into one effective experiment (Experiment 2, Table III).

Thus, there are now a total of 3 data sets, one for GuHCl+PI analytical extraction (28 days), one for GuHCl analytical extraction (84 days), and one for collagenase

digestion analytical extraction (84 days). The data from these three sets are summarized

in **Table V** for DBM and in **Table VI** for SB. **Figures 8** and **9** show the corresponding

plots. **Tables VII** and **VIII** summarize the statistical comparisons for the DBM and SB,

respectively.

TABLE V: BMP-7 CONTENT OF BOVINE DBM

Days in Buffer	Hours in Buffer	Analytical extraction method					
		GuHCl+PI		GuHCl		Collagenase	
		|Ave±SD| (ng/g DBM)	n	|Ave±SD| (ng/g DBM)	n	|Ave±SD| (ng/g DBM)	n
0.00	0	20.2±0.34	2	24.6±1.56	6	9.3±1.73	4
0.08	2	15.7±6.84	2	24.6±3.79	6	7.9±1.46	4
0.17	4	15.6±4.07	4	16.4±7.04	8	7.7±1.16	4
0.25	6	13.8±1.44	4	17.0±6.02	8	7.2±0.52	4
0.33	8	16.7±3.32	4	21.0±4.30	8	5.3±4.91	4
1.00	24	18.2±4.67	2	17.8±2.75	6	4.4±3.13	4
3.00	72	18.2±3.87	4	15.3±4.06	6	7.1±1.27	4
7.00	168	16.9±4.60	4	25.8±2.55	4	7.0±3.30	4
14.0	336	18.2±2.64	4	18.5±6.50	8	7.4±1.14	4
21.0	504	15.4±2.51	4	15.7±3.68	8	8.9±3.72	4
28.0	672	13.6±1.60	2	14.7±2.87	6	7.2±1.12	4
42.0	1008	N/A		11.8±2.18	4	5.4±0.25	4
56.0	1344	N/A		14.0±3.04	4	4.7±0.37	4
84.0	2016	N/A		13.8±2.16	4	4.4±0.43	4

TABLE VI: BOVINE BMP-7 CONTENT OF 40ml OF SB

| Days in Buffer | Analytical extraction method | | | | | |
| | GuHCl+PI | | GuHCl | | Collagenase | |
	|Ave±SD| (ng/g DBM)	n	|Ave±SD| (ng/g DBM)	n	|Ave±SD| (ng/g DBM)	n
0.00	0	0	0	8	0	0
0.08	10.1±1.12	4	9.7±2.17	8	6.8±0.99	4
0.17	8.3±0.35	4	10.6±2.66	8	6.7±2.13	4
0.25	8.5±0.71	4	9.3±2.39	8	8.7±0.90	4
0.33	13.3±5.26	4	9.5±4.34	8	9.6±1.25	4
1.00	6.1±2.68	4	11.2±4.88	8	11.4±1.18	4
3.00	15.4±6.85	4	16.9±9.89	8	16.4±0.97	4
7.00	18.4±2.93	4	34.3±23.6	8	24.9±2.22	4
14.0	11.8±3.60	4	36.6±27.4	8	21.1±19.5	4
21.0	12.8±5.46	4	63.5±46.8	8	55.6±1.37	4
28.0	14.4±5.47	4	59.7±42.7	8	21.9±20.5	4
42.0	N/A		113±10.3	4	93.2±3.61	4
56.0	N/A		119±6.3	4	94.6±4.69	4
84.0	N/A		146±36.7	4	95.9±5.77	4
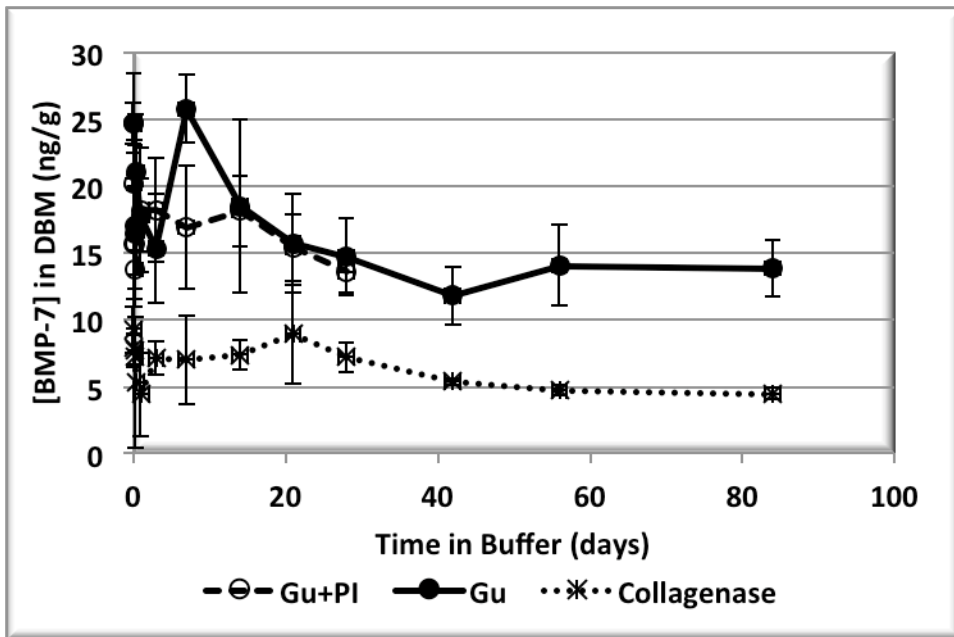
Figure 8. Plot of the concentration of bovine BMP-7 in DBM as a function of time in SB and the type of analytical extraction used. Ave±SD
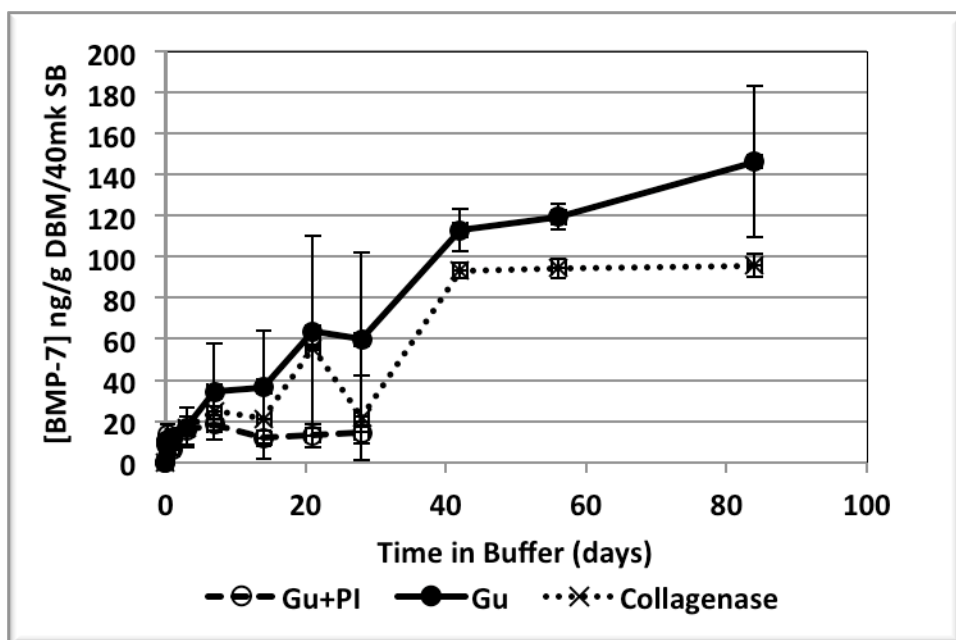
Figure 9. Plot of the concentration of bovine BMP-7 in SB as a function of time and the type of analytical extraction used. Ave±SD

TABLE VII: DBM BOVINE BMP-7 STATISTICAL ANALYSIS

Days in Buffer	GuHCl+PI |Ave±SD| (ng/g DBM)	n	GuHCl |Ave±SD| (ng/g DBM)	n	Collagenase |Ave±SD| (ng/g DBM)	n
0.00	20.2±0.34	2	24.6±1.56	6	9.3±1.73	4
28.0	13.6±1.60	2	14.7±2.87	6	7.2±1.12	4
84.0	N/A		13.8±2.16	4	4.4±0.43	4

Note: BMP-7 concentration at last extraction interval is significantly less than respective initial concentration. Initially, all three concentrations are different (p<0.05). At 28 days, GuHCl+PI and GuHCl regimens had greater concentrations than collagenase method (p<0.05)

TABLE VIII: SB BMP-7 STATISTICAL ANALYSIS. STUDENT-NEWMAN-KEULS TEST (p<0.05)

Days in Buffer	GuHCl+PI |Ave±SD| (ng/g DBM)	n	GuHCl |Ave±SD| (ng/g DBM)	n	Collagenase |Ave±SD| (ng/g DBM)	n
28.0	14.4±5.47	4	59.7±42.7	8	21.9±20.5	4
84.0	N/A		146±36.7	4	95.9±5.77	4

Note: At 28 days, BMP-7 concentration in GuHCl system is greater than the other systems (p<0.05). At 84 days, the concentration in the GuHCl system is greater than that in the collagenase system (p<0.05).

Regarding the DBM bovine BMP-7 profiles for the three analytical extraction methods, 1) the initial concentrations were all significantly different, 2) at 28 days, the concentrations for the GuHCl+PI and GuHCl methods were significantly greater than that for the collagenase method, and 3) by the last test interval, the concentrations for the three methods were each significantly less than their respective initial values. Regarding the statistical analysis for the SB BMP-7 profiles, 1) at 28 days the concentration for the GuHCl method was greater than that for the other two methods, and 2) at 84 days the concentration for the GuHCl method was greater than that for collagenase.

Importantly, comparison of the GuHCl and GuHCl+PI regimens showed that there was no overt evidence of enzymatic degradation occurring in the systems. Recall that for the latter, PI was not only included in the analytical extraction, but was also present in the SB that the DBM was exposed to in the extraction vessels. If enzymatic degradation was occurring, then the DBM

and SB BMP-7 profiles for the GuHCl+PI system would be greater than those for the GuHCl system, which was not the case. This provided justification for not including PI in the other runs, which simplified the preparative procedures.

Examination of **Table V** shows that, in general, there were 3 phases to the DBM BMP-7 profiles, i.e., an initial rapid decrease, a slow increase, and slower long-term decrease. However, the start and stop intervals for these 3 phases were not easily definable because of the sawtooth nature of portions of the DBM profiles, plus the large relative standard deviations in some of the measurements. Hence, for simplicity, the initial and final intervals for the 3 phases were somewhat arbitrarily defined as follows, Phase 1: 0-6 hr, Phase 2: 6-72 hr, and Phase 3: 72 hr to the final interval, whether that be 28 days or 84 days. Each phase was assumed to be linear, and the slopes, or zero-order rate constants, were computed for each phase using linear regression. The various zero-order rate constants are summarized in **Table IX** and illustrated in **Figure 10**.

TABLE IX: PHASE 1-3 RATE CONSTANTS FOR THE BOVINE DBM BMP-7 PROFILES

Phase	Time	Rate Constant (Ave±SEM) (ng BMP-7/g DBM/hr)		
		GuHCl+PI |Ave±SD|	GuHCl |Ave±SD|	Collagenase |Ave±SD|
1	0-6 hr	-0.967±0.306	-1.55±0.609	-0.32±0.0900
2	6-72 hr	0.0443±0.0363	-0.0554±0.0386	0.0148±0.0296
3	72 hr – 28 or 84 days	-0.00707±0.00227	-0.00362±0.00221	-0.00184±0.000567
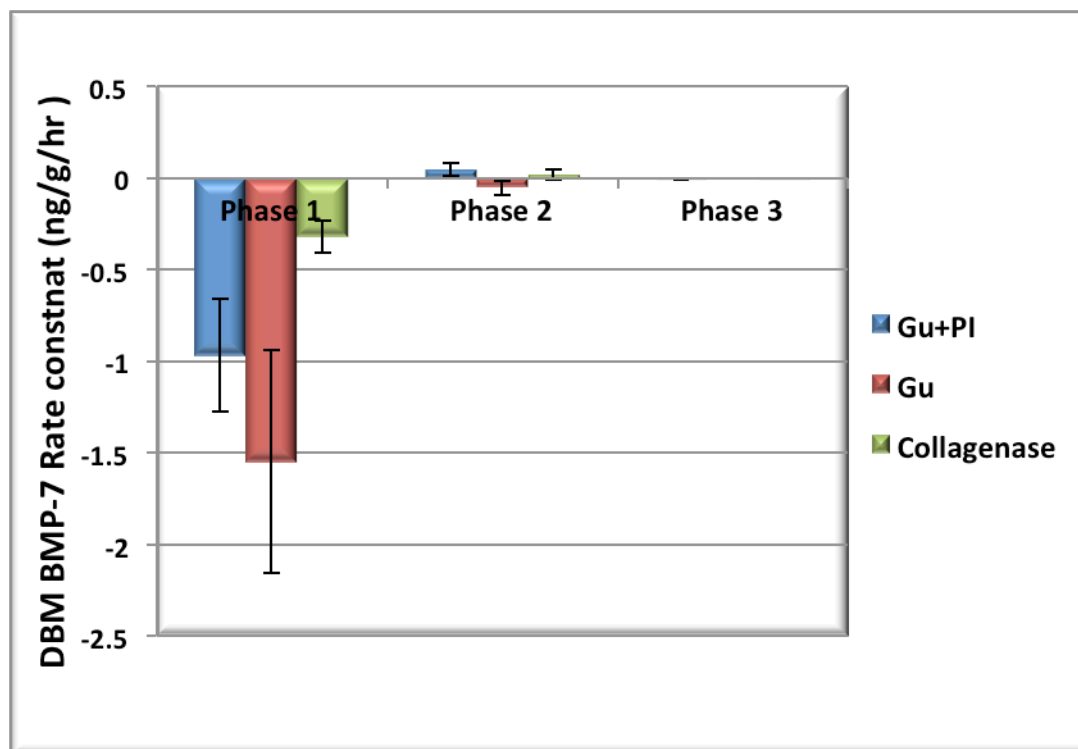
Figure 10. Zero-order rate constants for the bovine DBM BMP-7 profiles for each of the three analytical extraction methods. Ave±SEM.

These same intervals were used to define the three phases for the SB BMP-7 profiles. The first order rate constants for the SB BMP-7 profiles are summarized in **Table X** and **Figure 11.**

TABLE X: PHASE 1-3 RATE CONSTANTS FOR THE SB BMP-7

Phase	Time	Rate Constant (Ave±SEM) (ng BMP-7/g DBM/hr)		
		GuHCl+PI |Ave±SD|	GuHCl |Ave±SD|	Collagenase |Ave±SD|
1	0-6 hr	1.18±0.922	1.44±0.990	1.3±0.500
2	6-72 hr	0.0783±0.081	0.116±0.00166	0.112±0.00717
3	72 hr – 28 or 84 days	0.00510±0.00526	0.0686±0.00733	0.0476±0.0114
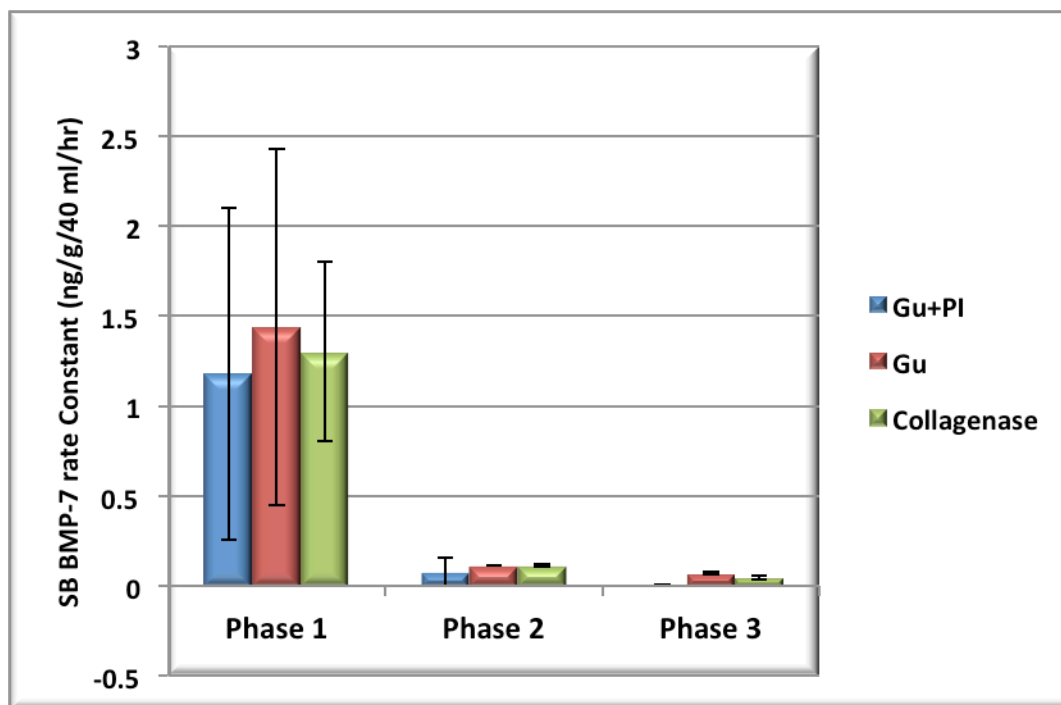
Figure 11. Zero-order rate constants for the SB bovine BMP-7 profiles for each of the three analytical extraction methods. Ave±SEM.

As can be seen from **Tables IX** and **X** and **Figures 10** and **11**, the most rapid changes in the measured BMP-7 concentrations in the DBM and SB occurred during Phase I, with the rate constants for Phases 2 and 3 approximately one to two orders of magnitude smaller.

Table XI compares the overall reduction in measured DBM BMP-7 for the three datasets as well as the corresponding increases in SB BMP-7 content. A student t-test was used to determine whether the net change in BMP-7 content of the systems was significant ($p<0.05$).

TABLE XI: COMPARISON OF NET CHANGE IN BOVINE BMP-7 CONTENT OF THE DATA SETS

	Interval	GuHCl+PI (ng/g/40ml) |Ave±SD|	GuHCl (ng/g/40ml) |Ave±SD|	Collagenase (ng/g/40ml) |Ave±SD|
DBM	Initial	20.2±0.34 (n=2)	24.6±1.56 (n=6)	9.30±1.73 (n=4)
	Last interval	13.6±1.60 (n=2)	13.8±2.16 (n=4)	4.40±0.43 (n=4)
	Difference	-6.60±1.64 (n=2*)	-10.8±2.66 (n=4*)	-4.90±1.78 (n=4*)
SB	Initial	0	0	0
	Last interval	14.4±5.47 (n=4)	146±36.7 (n=4)	95.9±5.77 (n=4)
	Difference	14.4±5.47 (n=4)	146±36.7 (n=4)	95.9±5.77 (n=4)
Net Change		7.8±5.71 (p=0.193)	135.2±36.8 (p<0.001)	91.0±6.04 (p<0.001)

Note: * is "effective" n value.

Specifically, regarding DBM, the final (28 or 84 day) BMP-7 content was subtracted from the corresponding initial content to determine the apparent reduction in DBM BMP-7 content. Since the initial and final values were expressed as a mean±SD, the difference must be expressed in the same way. To do this, the means are subtracted, e.g., in the case of GuHCl+PI, this would be 20.2-13.6 = 6.60 ng/g. To determine the standard deviation of the difference, this is equal to the square root of the sum of the squares of the individual standard deviations, e.g., $Sqrt(0.34^2 + 1.60^2) = 1.64$. Thus, for the GuHCl+PI group, there was an overall loss of 6.60±1.64 ng/g BMP-7. The net loss in DBM BMP-7 content for the other systems, as well as the net increase in BMP-7 content for the SB systems (per 40 ml) were calculated similarly. As can be seen from the last row in **Table XI**, the BMP-7 content of the SB increased a greater amount than did the loss of BMP-7 from the DBM. A t-test was used to determine the significance of differences between the reduction of DBM BMP-7 and the gain of SB BMP-7 in a given experiment. Note that for the DBM BMP-7 values, the number of readings must be considered. For the GuHCl+PI

and collagenase experiments, the DBM n values were 2 and 4, respectively. For the GuHCl experiment, the DBM n value we will use will be 4 since that is "worst case" from a statistical perspective. Hence, "effective" n values are listed in **Table XI** for the DBM BMP-7 difference values. Thus, 1) all three systems had a greater increase in the final SB BMP-7 content than could be accounted for by the corresponding decrease in DBM BMP-7 contents, and 2) this increase was significantly greater than the decrease for the GuHCl and collagenase systems only.

2. Bonus Results and Analysis

Weight of the Bonus DBM (BDBM) logs used, and raw data obtained from ELISA readings can be found in **Appendix E**. **Table XII** and **Figure 12** show the concentrations of BMPs-2, -4, and -7 in BDBM and SB vs. time.

TABLE XII: [BMPs-2, -4, -7] IN BONUS DBM AND RESPECTIVE SB VS. TIME

Time (hr)	BMP-2		BMP-4		BMP-7	
	[BMP-2] in BDBM (ng/g) |Ave±SD|	[BMP-2] in SB (ng/g/40ml) |Ave±SD|	[BMP-4] in BDBM (ng/g) |Ave±SD|	[BMP-4] in SB (ng/g/40ml) |Ave±SD|	[BMP-7] in BDBM (ng/g) |Ave±SD|	[BMP-7] in SB (ng/g/40ml) |Ave±SD|
0	28.1 ± 1.3	0 ± 0	0.577 ± 0.056	0 ± 0	92.9 ± 7.5	0 ± 0
2	36.3 ± 3.5	1.37 ± 0.346	0.882 ± 0.042	Not detected	101 ± 5.9	12.0 ± 0.16
4	37.9 ± 3.4	0.90 ± 0.349	1.08 ± 0.085	Not detected	102 ± 1.7	12.1 ± 0.296
6	37.1 ± 1.7	1.63 ± 0.171	0.94 ± 0.1	0.000778 ± 0	103 ± 13	11.7 ± 0.708
8	33.7 ± 1.7	1.50 ± 0.236	0.869 ± 0.135	Not detected	107 ± 7.6	13 ± 0.154
24	37.8 ± 1.4	2.15 ± 0.318	0.902 ± 0.086	0.00296 ± 0.00345	101 ± 6.8	15.3 ± 1.6
72	34.5 ± 2.5	2.67 ± 0.260	0.704 ± 0.077	0.0194 ± 0.00635	102 ± 4.3	17.3 ± 1.39
168	28.5 ± 0.7	3.13 ± 0.204	0.552 ± 0.063	0.0227 ± 0.00107	100 ± 10.3	27.2 ± 2.73
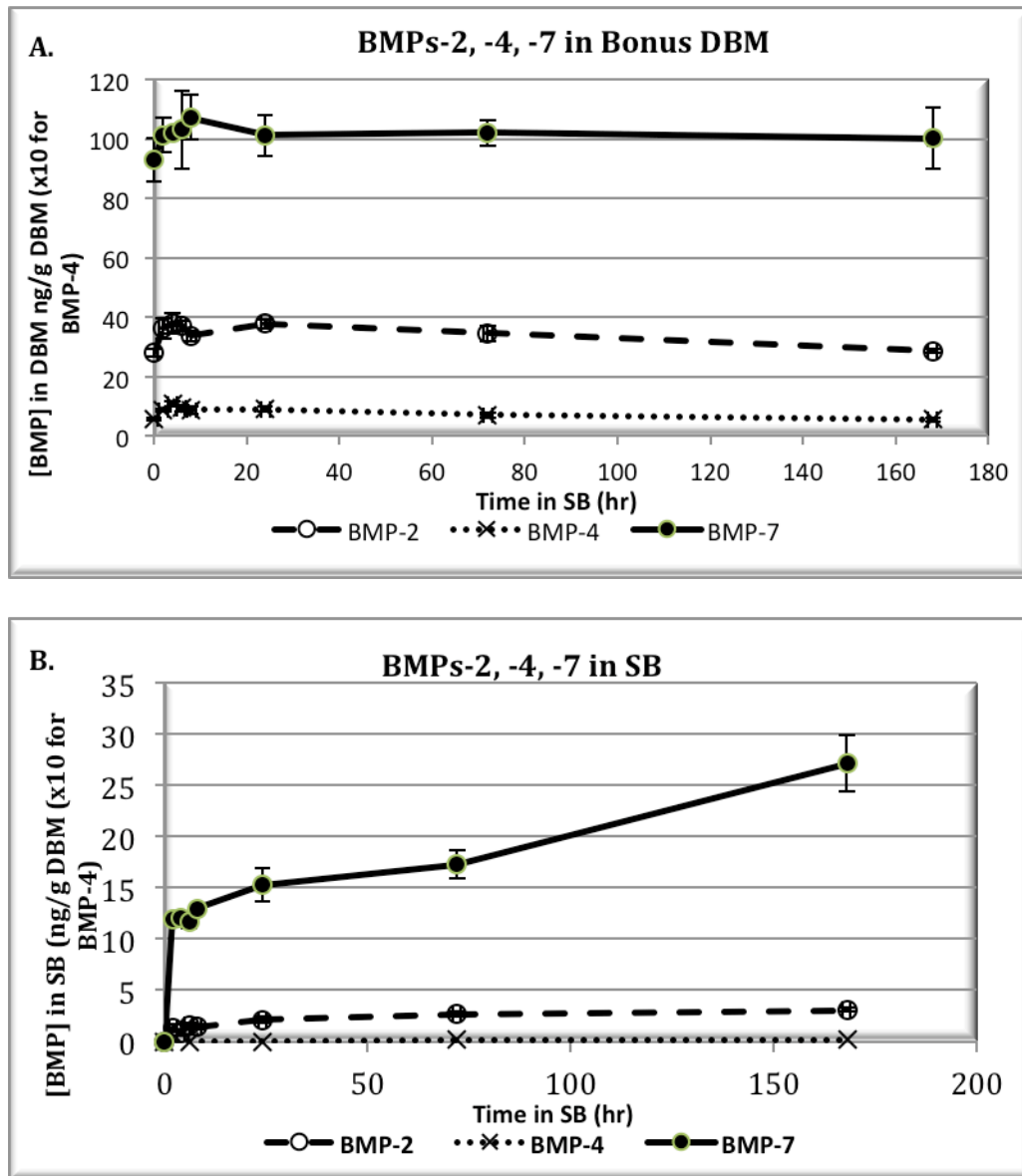
Figure 12. A. [BMPs-2, -4, -7] remaining in BDBM over a period of 7 days of hydration in SB. B. [BMPs-2, -4, -7] eluted in SB from BDBM over a period of 7 days.

The hydrated BDBM cylinders completely dispersed in the SB within 60 minutes of rocking. The initial concentrations in the BDBM were 28.1±1.3, 0.58±0.06, and 92.9±7.5 ng/g BDBM (ave±SD) for BMP-2, -4, and -7, respectively. The final concentrations after 7 days were

28.5±0.7, 0.55±0.06, and 100±10.3 ng/g BDBM, respectively. There were no significant changes in any BMP concentrations in BDBM between the 0 and 7-day values. Also, for all BMP types, their concentrations in BDBM appeared to peak after 4-8 hours, in the range of 15% to 60% above their respective initial values. There was a biphasic increase in the SB BMP content, initially quickly then more slowly. After 7 days, the amounts of BMP-2, -4, and -7 in the SB were 3.1±0.2, 0.023±0.001, and 27.2±2.7 ng/g BDBM (ave±SD), respectively. The small amount of BMP-4 in SB was difficult to detect for some of the shorter intervals.

Relative to the initial measured BMP-2, -4, and -7 content in BDBM, 11%, 4%, and 29% of these BMPs were released into SB by 7 days, respectively. However, by 7 days there was no significant change from their initial concentrations in BDBM. The SB BMP concentration profiles were modeled as 2 linear segments, i.e., from 0 to 8 hr and 8 to 168 hr. Zero-order rate constants were estimated for BMP-2 and -7 release but not for BMP-4 release due to measurement difficulties. The early and late rate constants for BMP-2 release were 0.16±0.08 and 0.0089±0.003 ng/g BDBM/hr (ave±SE), respectively, and for BMP-7 were 1.29±0.67 and 0.086±0.009 ng/g BDBM/hr, respectively. Corresponding zero-order rate constants were not computed for the BDBM BMP profiles because the nature of the profiles did not lend themselves to such analysis.

B. DISCUSSION

1. Bovine and Bonus DBM Results

Although there is a significant amount of literature regarding measurement of BMPs in DBM, either for screening potential donors [34, 47-49] or for comparison of commercial

DBM grafts [50-51], to the best of our knowledge, Pietrzak, et al. (27), is the only cited literature that investigates BMP elution from DBM. They examined the elution of BMP-7 from particulate bovine DBM for 7 days, using the same test methods as used in the current study, including GuHCl analytical extraction. They had three important observations. First, the concentration of BMP-7 in SB continued to increase over the course of 7 days, suggesting longer-term studies would be required to fully characterize the release profile. Second, there was an anomalous dip, or minimum, in the DBM BMP-7 profile at about 4-6 hours. This was difficult to explain on the basis of mass transfer theory. Third, a mass balance (BMP-7 in DBM and in SB) indicated that there was more BMP-7 in the closed extraction tubes after 7 days than there was initially. They proposed that BMPs are associated with DBM, at least conceptually, in two compartments differing by the tenacity by which the BMPs are bound to the matrix, i.e., the loose compartment and the tight compartment (**Figure 13**). Further, they suggested that these unexpected findings could be due to the relative size of these two compartments, as well as the magnitude of the rate constants associated with intra-compartmental transfer of BMPs and the transfer of BMPs to the surroundings. In effect, they proposed that BMPs can only elute to the surroundings, or be extracted with guanidine-HCl, from the loose compartment. As BMPs elute into SB, the tight compartment would function as a BMP reservoir, replenishing the loose compartment with BMPs over time. Thus, upon exposure to an aqueous environment in vivo, there would be an initial rapid release of BMPs from the loose compartment to jumpstart osteoinduction. Then there would be a slower, sustained release to maintain the effect as mediated by the stores of BMPs in the tight compartment. Insufficient data was available, however, for them to estimate values for these rate constants. Finally, their results implied that guanidine-HCl does not extract BMPs

from DBM with 100% efficiency, but rather, its ability to extract BMP depends on the

hydration history of the DBM. This had never been proposed before.
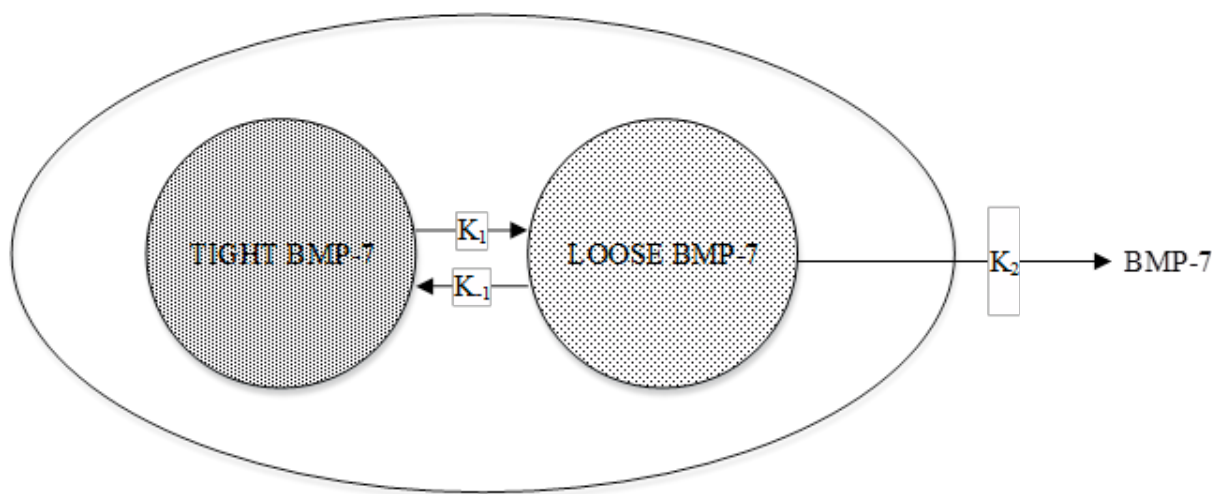
Figure 13. Schematic of the proposed 2-compartment model of the association of BMP-7 with bone matrix. k_1 and k_{-1} are the rate constants characteristic of the transfer of BMP-7 from the tight to the loose compartment and the elution of BMP-7 from the loose compartment, respectively. K_2 is the rate constant associated with the transfer of BMP-7 from the loose compartment to the surroundings.

How do the results of the current study compare to these three observations of Pietrzak, et al (27), described above? The bovine studies showed the concentration of BMP-7 increasing in the SB for at least 42 days. For the human DBM studies, the BMP-2 and BMP-7 concentrations in the SB were still increasing at 7 days, corroborating Pietzak, et al (27). Regarding the human BMP-4 study, very little BMP-4 was detectable at any of the time points so conclusions cannot be drawn regarding the completion of its release by 7 days.

The observed dip in the BMP-7 profile of Pietrzak, et al (27), was one of the most perplexing aspects of their study. In the current study, a dip was observed in the bovine DBM BMP-7 profiles for both the guanidine-HCl and collagenase analytical extraction systems at 72 hours and 24 hours, respectively. By contrast, there was no apparent dip in the human Bonus II DBM BMP profiles for BMP-2, BMP-4, or BMP-7. Finally, a mass balance showed that for the bovine DBM systems utilizing both guanidine-HCl and collagenase for the analytical extraction, the increase in BMP-7 content of the SB exceeded the apparent BMP-7 decrease in the DBM, showing that more BMP-7 was present in the extraction vessels at the end of the study than was present initially. This was essentially the case with the Bonus II DBM studies as well since there was virtually no difference in the measured BMP-2, BMP-4, and BMP-7 content in the DBM at the end of the 7 days study compared to the initial values, yet measureable quantities of these BMPs were present in the SB, increasing over the course of the 7 days.

One important difference to note between the bovine and Bonus II DBM studies was that in the case of the latter, there was no initial rapid decrease in BMP concentration in the DBM to correlate with the rapid initial increase in BMP concentration in the SB. To the contrary, the measured BMP content of the Bonus II DBM actually increased for the first 2 hours during the time that the BMP concentration in the SB was also increasing. The reason for this difference between the bovine and Bonus II DBM BMP profiles is unknown, but an effect of the carrier in the latter cannot be ruled out.

To better quantify the rates of change in BMP concentrations in the SB and DBM, the profiles were typically divided into two or three consecutive linear segments, with linear regression performed for each segment to determine its slope. This was not done, however, for the Bonus II BMP-2, BMP-4, and BMP-7 DBM profiles because there was little overall change in these concentrations over the course of the 7-day study. **Tables XIII** and **XIV** show these linear slopes, or rates, for the SB and DBM BMP profiles, respectively. Note that the rates of increase of BMP-7 in the SB for the bovine DBM systems from the current study are listed as a range corresponding to both systems without PI, i.e., utilizing guanidine-HCl and collagenase as the analytical extractant since the extraction vessels with SB and DBM were set up identically in both cases. Also included are rates of increase of BMP-7 in the SB from Pietrzak, et al (27), which are listed as a range corresponding to the three DBM particle size ranges they examined.

Table XIII: SUMMARY OF BMP SB RATE CONSTANTS IN CURRENT STUDY AND PIETRZAK, ET AL (27)

Study	DBM Source	SB BMP rate constants (ng/g/hr) Ave±SD		
		BMP-2	BMP-4	BMP-7
Current	Bovine			0-6 hr: 1.30±0.50 to 1.44±0.99 6-72 hr: 0.11±0.01 to 0.12±0.00 3-84 day: 0.05±0.01 to 0.07±0.01
Current	Human	0-8 hr: 0.16±0.08 8 hr–7day:0.01±0.00	N/A	0-8 hr: 1.29±0.67 8 hr-7 day: 0.09±0.01
Pietrzak, et al [33]	Bovine			0-8 hr: 2.2-3.3 8 hr – 7 day: 0.04-0.15

Table XIV: SUMMARY OF BMP DBM RATE CONSTANTS IN DBM IN CURRENT STUDY

Study	DBM Source	DBM BMP rate constants (ng/g/hr) Ave±SD			Comments
		BMP-2	BMP-4	BMP-7	
Current - GuHCl	Bovine			0-6 hr: -1.55±0.609 6-72 hr: -0.055±0.039 3day-84 day: -0.004±0.002	Minimum reached at 72 hr. Long-term reduction begins at 7 days,
Current - Collagenase	Bovine			0-6 hr: -0.32±0.09 6-72 hr: 0.015±0.030 3day-84 day: -0.002±0.001	Minimum reached at 24 hr. Long-term reduction begins in 21 days.
Current	Human	N/A	N/A	N/A	No minimum observed for any BMP type. No change in initial and final BMP concentration. BMP concentrations peaked between 0 and 7 days.

From Pietrzak, et al (27), the initial rate of increase of bovine BMP-7 concentration in the SB was in the range of $2.2 - 3.3$ ng/g/hr, depending on the DBM particle size. In the current study, the mean rate of bovine BMP-7 increase in the SB was $1.3 - 1.4$ ng/g/hr while the rate of increase of human BMP-7 from Bonus II into SB was 1.29 ng/g/hr. Given the crude means of estimating these rates, they can be viewed as similar. After the initial 6-8 hours of release, the rate of increase of human or bovine BMP-7 in SB decreased by 1-2 orders of magnitude from its initial value. By comparison, the intial rate of increase of human BMP-2 in SB from the Bonus II DBM studies was only about 10% of the corresponding initial rate of increase of human BMP-7 in SB. However, the initial concentration of human BMP-2 in Bonus II was only about one-quarter of that of human BMP-7, which undoubtedly contributed to this. Similarly, the initial concentration of human BMP-4 in Bonus II DBM was less than 1% the initial concentration of

human BMP-7 and the initial rate of its increase in SB could not even be measured. In terms of the initial change of bovine BMP-7 concentration in DBM in the current study, both guanidine-HCl and collagenase analytical extraction yielded initial rates of -1.55 and -0.32 ng/g/hr, respectively. While initial rates of change of the concentrations of human BMP-2, BMP-4, and BMP-7 were not measured in the current study, the initial rates were positive, i.e., the concentration of these BMPs in the Bonus II DBM appeared to increase, even while their concentration was increasing in the SB as well. This is a fundamental difference in the Bonus II DBM BMP profiles compared to the bovine DBM BMP-7 profiles.

Regardless of the reason for the apparent dip in the DBM BMP-7 profiles of the bovine DBM systems, one would expect that the measured BMP-7 content of the DBM would eventually become zero as all of the BMP-7 eluted out of the DBM into the SB. By 7 days Pietrzak, et al (27), found no evidence of the BMP-7 content of the bovine DBM diminishing over time after the early dip in the profile. In the current study, there was evidence that by approximately 7 to 21 days, the bovine DBM BMP-7 concentration began a long-term decline for both the guanidine-HCl and collagenase analytical extraction studies. However, by 84 days exposure to SB, both of the systems still had a measured BMP-7 concentration in the DBM that was about half the initial value. So even this extended period of extraction in SB was not of sufficient duration to remove all of the BMP-7 from the DBM. Regarding the human BMP-2, BMP-4, and BMP-7 profiles in Bonus II DBM, there was no definitive trend toward a reduction in the concentration in DBM since the concentration at 7 days was essentially the same as it was initially.

Pietrzak, et al (27), suggested that one reason there appeared to be more BMP-7 present in the extraction vessels over the 7 day interval (not constant over time) was that the guanidine-HCl analytical extraction method was incapable of removing 100% of the BMP-7 at each time point. They reasoned that if it was capable of this, then the total amount of measured BMP-7 in the systems would have been constant over time. Recall that guanidine-HCl functions by denaturing the BMPs and disrupting their native association with bone matrix. To the extent that there may be two DBM compartments with which BMPs reside and that water may affect the distribution of BMPs between these two compartments, it is plausible that all of this could give rise to the apparent early dip in DBM BMP concentration as well as the observed changes in the total amount of BMPs present in the system over time. By contrast, collagenase digests the collagen bone matrix, thereby freeing the bound BMPs for measurement by ELISA. This being the case, one would expect that all of the BMPs associated with DBM at each time point would be measureable and that the total amount of BMPs present in the DBM/SB systems would be constant over time. That this wasn't the case indicates that this unusual phenomenon is not uniquely associated with a particular analytical extraction method, but manifests itself with two quite different methods. Another interesting comparison is that the collagenase method yielded only about half the bovine DBM BMP-7 content as the guanidine-HCl method throughout the course of the entire 84 day extraction in SB in the current study. Since it is reasonable to view the collagenase method as the more robust way of releasing BMPs from DBM, this finding is contrary to expectation. While it is possible that collagenase might partially degrade BMPs, this is unlikely since enzymes are typically highly specific for their substrate. Perhaps during collagenase digestion of bone matrix, small oligomers of collagen remain associated with the released BMPs and these might interfere with the ELISA assay. Also, the collagenase analytical

extraction was performed at 37° C while the guanidine-HCl extraction was performed at 4° C. Perhaps the BMP molecules are less stable at the higher temperature.

As described above, the Bonus II human DBM product contains a carrier that consists of thermally denatured human DBM prepared from the same donor that supplied the active DBM. Once that product is hydrated, the carrier gives the material the handling characteristics of a putty to facilitate placement in a bone defect. When the hydrated product was placed in the SB and shaken in the extraction vessel, the product eventually dispersed in the medium. Following extraction in SB, the vessels were centrifuged to separate the DBM pellet from the SB supernatant, unlike the case in the bovine systems where carrier was not present and gravity was sufficient to form a pellet. This shows that the SB/carrier combination was too viscous for the DBM pellet to settle out without centrifugation. When the extraction vessels were centrifuged, at least a portion of the denatured carrier DBM became part of the pellet. It is possible that when the carrier DBM was dispersed in the SB during shaking, some of the BMPs that released from the active DBM into the SB became associated with the carrier DBM. Then, upon centrifugation, a hybrid pellet would have formed, i.e., active DBM with residual BMPs remaining within it and carrier DBM with eluted BMPs associated with it. During the analytical extraction of lyophilized Bonus II DBM pellet with guanidine-HCl, BMPs would likely be released from both DBM moieties. Perhaps this was a contributing factor in the lack of an observed initial decline in the Bonus II DBM concentration of BMP-2, BMP-4, and BMP-7.

Others have shown a complex relationship between BMPs and bone matrix as well. For instance, Sampath and Reddi (25) demonstrated that bone inductive proteins are associated with

multiple compartments within bone (25). They found that about 25% of the total biologic activity of bone induction was associated with cells and not masked by bone mineral, about 15% was associated with the mineral phase, and the remaining was associated with the collagen matrix. Han, et al (31), proposed that the association of BMPs with DBM includes free, loosely bound, and tightly bound states. Pietrzak, et al (27) suggested the hydration history of DBM may influence the ability of guanidine-HCl to extract BMPs from DBM which could partially account for some of the unusual characteristics of the BMP profiles. This change in BMP association with DBM may be related to the collagen/growth factor association disruption by heat and water exposure (31). Water contributes to the conformational change of the collagen matrix induced by heat and accelerates the release of BMPs (31). Under hydrous conditions, collagen can be unstable at or even well below body temperature (37°C), since the thermal helix-coil transition of collagen is associated with the degree of hydration (52).

During the analytical extraction procedures for both the GuHCl and collagenase method, it is possible BMPs can be extracted more efficiently from the DBM with a longer hydration history due to increased swelling and loosening of the matrix which frees more BMPs once they are denatured and more efficiently digests the collagen with the help of previous disruption of the collagen by water exposure; respectively. If this is the case, our study supports the "two-compartment model" hypothesized by Pietrzak et al, which would be dependent on the collagen association of BMPs during DBM hydration; the "loose" compartment describing BMPs in the swollen collagen as hydration progresses, and the "tight" compartment describing BMPs within dry or minimal hydrated DBM.

So, what has been accomplished by this study? 1) It showed that there was no overt evidence of proteases degrading BMPs, at least in the bovine DBM systems tested. 2) The elution of BMPs from DBM in an aqueous environment occurs over a period of several weeks to months, which is longer than would be expected to be necessary to effect bone repair. 3) This was the first study to measure, and attempt to analyze, the elution of human BMPs from a human DBM bone graft. 4) The unusual bovine BMP profile characteristics reported by Pietrzak, et al (27), including the anomalous dip in the DBM BMP-7 profile and the apparent net increase in total BMP-7 present in the closed extraction systems over time were reaffirmed . This appeared to be independent of whether guanidine-HCl or collagenase was used for the analytical DBM extraction. The latter characteristic was also reaffirmed in the human Bonus II DBM studies. 5) The DBM and SB profiles of human BMP-2, BMP-4, and BMP-7 in the Bonus II DBM systems were qualitatively similar to each other, differing mainly in magnitude which appeared to be related to the initial concentrations of these individual BMPs in the DBM. 6) It is possible that some of the differences in the BMP profiles between the human and bovine DBM systems could be attributed to the presence of a carrier in the former.

There were several hypotheses that were proposed at the beginning of this study and it is instructive to determine if they were shown to be true or false. First, it was hypothesized that the BMP-7 would completely elute out of the bovine DBM by 12 weeks because mineralization of the extracellular matrix is largely complete by that time (11) which would seem to make longer-term elution biologically unnecessary. The elution of BMP-7 from bovine DBM was tested for 12 weeks, but it was apparent that the rate of increase of BMP-7 in SB was significantly diminishing by that time. Based on the shape of the SB BMP-7 profile, the rate of elution of

BMP-7 from the bovine DBM into SB would likely have been very small by 12 weeks, suggesting that this was a reasonably hypothesis although it cannot be stated with certainty that it is true. Second, it was hypothesized that collagenase would be more efficient at releasing BMPs from DBM since it digests the substrate to which the BMPs are associated, thus showing a simpler, exponential decrease in DBM BMP-7 over time in SB. Experimentally, however, it was shown that the collagenase method of analytical extraction yielded bovine DBM/BMP-7 profiles that were very similar to that obtained using guanidine-HCl for the analytical extraction. Thus, this hypothesis was false. Finally, the elution profiles of human BMPs-2, -4, and-7 from Bonus II DBM were hypothesized to be similar to each other because these molecules share many features in common. This was shown to be the case, with the various BMP profiles all qualitatively similar, differing in magnitude in a similar fashion to their initial concentrations in the DBM.

2.   Limitations

There were some limitations to this study. First, the longest-term elution was 84 days for the bovine DBM/BMP-7 studies. This interval was not sufficient to demonstrate the completion of BMP elution which appeared to remain ongoing at that time. Similarly, a 7-day interval was insufficient to demonstrate complete elution of BMP-2, BMP-4, and BMP-7 from the human Bonus II DBM. Second, the SB was not an infinite sink, which could have hindered release of BMPs from DBM during the later time intervals as the concentration of BMPs in SB increased. Third, the entire study was performed at room temperature (25-27° C) rather than body temperature (37° C). It is possible BMP elution characteristics may be temperature dependent. Han, et al (31), provided evidence that growth factor release increases with increasing temperature based on bioassay; however, this has not been confirmed by direct measurement

such as ELISA. Fourth, a broad, but single, DBM particle size range was used for all of the experiments; stratifying the DBM particles into narrow, discrete ranges, and testing them individually would have allowed determination of the release rate as a function of particle size. Fifth, this was an in vitro system which had many important differences with the in vivo environment, including lower extraction temperature, the absence of an enzymatic and cellular DBM degradation mechanism, and the inability to form new bone. As such, the degree to which the BMP release behavior observed in this study represents what occurs in vivo is unknown. When DBM is implanted in a living organism, BMP release causes new bone to form through both osteoinduction and osteoconduction. As this occurs, the DBM eventually degrades and is removed while new bone fills in the area. Thus the living system is much more dynamic than the in vitro system. It is possible that as the DBM particles degrade in vivo, their degradation would accelerate BMP release. However, as new bone forms and becomes deposited in the region, the DBM particles may become encapsulated in new bone which could then inhibit the further release of BMPs from the DBM. Nevertheless, this in vivo model system does provide insight into what may occur in living systems.

IV.   Future work

To the extent that the anomalous DBM BMP profiles were due to the inability of the analytical extraction methods to completely remove all residual BMPs from the DBM, further develop of such methods could be beneficial. Also, it would be interesting to combine in vitro with in vivo work. For instance, the DBM particles could be extracted in SB for various lengths of time and then implanted in an animal model to determine the relationship between in vitro extraction and in vivo new bone formation. In this manner, the amount of new bone that formed would be a biological assay of the remaining activity of the extracted DBM.

V. CONCLUSION

This was the longest-term, most detailed study of the elution of BMPs from DBM that has ever been conducted which is relevant to an understanding of the osteoinductive process as well as basic bone biology. It showed that such elution, at least under the conditions examined, occurs over a period of weeks to months, that there are some anomalous aspects of the elution which defy simple explanation, and that improved ways to extract residual BMPs from DBM for measurement may be required to more fully understand the process.

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APPENDIX A

Instructions Included with DY354 ELISA Kit

DY354

MATERIALS PROVIDED
Bring all reagents to room temperature before use.

Capture Antibody (Part 840971, 1 vial) - 360 µg/mL of mouse anti-human BMP-7 when reconstituted with 1.0 mL of PBS. After reconstitution, store at 2 - 8" C for up to 60 days or aliquot and store at -20" C to -70" C in a manual defrost freezer for up to 6 months.2 Dilute to a working concentration of 2.0 µg/mL in PBS,3 without carrier protein.

Detection Antibody (Part 840972, 1 vial) - 90 µg/mL of biotinylated mouse anti-human BMP-7 when reconstituted with 1.0 mL of Reagent Diluent (see Solutions Required section). After reconstitution, store at 2 - 8" C for up to 60 days or aliquot and store at -20" C to -70" C in a manual defrost freezer for up to 6 months.2 Dilute to a working concentration of 0.5 µg/mL in Reagent Diluent with 2% heat inactivated normal goat serum (NGS). Prepare 1 - 2 hours prior to use.

Standard (Part 840973, 1 vial) - 80 ng/mL of recombinant human BMP-7 when reconstituted with 0.5 mL of Reagent Diluent (see Solutions Required section). Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Store reconstituted standard at 2 - 8° C for up to 60 days or aliquot and store at -70° C for up to
6 months.2 A seven point standard curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 4000 pg/mL is recommended.

Streptavidin-HRP (Part 890803, 1 vial) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Store at 2 - 8° C for up to 6 months after initial use.2 DO NOT FREEZE. Dilute to the working concentration specified on the vial label using Reagent Diluent (see Solutions Required section).

SOLUTIONS REQUIRED
PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2 - 7.4, 0.2 µm filtered.

Wash Buffer - 0.05% Tween# 20 in PBS, pH 7.2 - 7.4
(R&D Systems Catalog # WA126).

APPENDIX A (Continued)

Reagent Diluent - 1% BSA4 in PBS, pH 7.2 - 7.4, 0.2 μm filtered (R&D Systems Catalog # DY995). Quality of BSA is critical (see Technical Hints).

Substrate Solution - 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems Catalog # DY999).

Stop Solution - 2 N H2SO4 (R&D Systems Catalog # DY994). Tween is a registered trademark of ICI Americas.

GENERAL ELISA PROTOCOL (DY354)

Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 μL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.

2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.

3. Block plates by adding 300 μL of Reagent Diluent to each well. Incubate at room temperature for a minimum of 1 hour.

4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 μL of sample or standards in Reagent Diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.

2. Repeat the aspiration/wash as in step 2 of Plate Preparation.

3. Add 100 μL of the Detection Antibody, diluted in Reagent Diluent with NGS, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.

4. Repeat the aspiration/wash as in step 2 of Plate Preparation.

APPENDIX A (Continued)

5. Add 100 μL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

6. Repeat the aspiration/wash as in step 2.

7. Add 100 μL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

8. Add 50 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

APPENDIX B

TABLE XV: GuHCl+PI: [BMP-7] IN BUFFER-ELUTED BOVINE DBM

Time (hr)	DBM Weight in GuHCl (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
0	0.298	608.681	20425.537	20.426
0	0.298	594.203	19939.698	19.940
2	0.305	330.251	10827.902	10.828
2	0.305	625.245	20499.836	20.500
4	0.300	536.474	17882.467	17.882
4	0.300	434.941	14498.033	14.498
4	0.302	594.139	19673.477	19.673
4	0.302	314.964	10429.272	10.429
6	0.300	369.495	12316.500	12.317
6	0.300	472.321	15744.033	15.744
6	0.303	407.117	13436.205	13.436
6	0.303	409.263	13507.030	13.507
8	0.298	536.536	18004.564	18.005
8	0.298	600.249	20142.584	20.143
8	0.304	373.966	12301.513	12.302
8	0.304	492.176	16190.000	16.190
24	0.297	637.190	21454.209	21.454
24	0.297	440.877	14844.343	14.844
72	0.299	398.200	13317.726	13.318
72	0.299	607.632	20322.140	20.322
72	0.301	513.188	17049.435	17.049
72	0.301	665.057	22094.917	22.095
168	0.304	647.829	21310.164	21.310
168	0.304	610.452	20080.658	20.081
168	0.302	442.761	14660.960	14.661
168	0.302	347.785	11516.060	11.516
336	0.301	615.452	20446.910	20.447
336	0.301	611.306	20309.169	20.309
336	0.305	506.825	16617.213	16.617
336	0.305	463.846	15208.066	15.208
504	0.301	401.845	13350.332	13.350
504	0.301	536.972	17839.601	17.840
504	0.299	516.502	17274.314	17.274
504	0.299	391.792	13103.411	13.103
672	0.307	381.602	12430.033	12.430
672	0.307	451.263	14699.121	14.699

APPENDIX B (Continued)

TABLE XVI: GuHCl+PI: [BMP-7] IN BUFFER

Time (hr)	DBM Weight in SB (g)	ELISA Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/40ml)
2	1.504	429.657	285.676	11.427
2	1.504	372.934	247.961	9.918
2	1.501	327.636	218.278	8.731
2	1.501	383.902	255.764	10.231
4	1.498	314.680	210.067	8.403
4	1.498	326.272	217.805	8.712
4	1.501	302.580	201.586	8.063
4	1.501	297.182	197.989	7.920
6	1.504	318.429	211.721	8.469
6	1.504	348.754	231.884	9.275
6	1.501	284.388	189.466	7.579
6	1.501	324.282	216.044	8.642
8	1.502	205.022	136.499	5.460
8	1.502	586.056	390.184	15.607
8	1.498	577.381	385.435	15.417
8	1.498	624.249	416.722	16.669
24	1.501	119.355	79.517	3.181
24	1.501	357.770	238.354	9.534
24	1.506	189.397	125.762	5.030
24	1.506	251.376	166.916	6.677
72	1.503	316.668	210.691	8.428
72	1.503	485.292	322.882	12.915
72	1.502	582.965	388.126	15.525
72	1.502	927.465	617.487	24.699
168	1.502	588.766	391.988	15.680
168	1.502	611.635	407.214	16.289
168	1.504	733.132	487.455	19.498
168	1.504	825.709	549.009	21.960
336	1.503	241.945	160.975	6.439
336	1.503	493.266	328.188	13.128
336	1.508	539.714	357.901	14.316
336	1.508	498.664	330.679	13.227
504	1.503	192.087	127.802	5.112
504	1.503	650.469	432.780	17.311
504	1.505	481.860	320.173	12.807
504	1.505	600.901	399.270	15.971
672	1.500	361.198	240.799	9.632
672	1.500	834.020	556.013	22.241
672	1.510	499.758	330.966	13.239
672	1.510	467.184	309.393	12.376

APPENDIX C

TABLE XVII: GuHCl RUN 1: [BMP-7] IN BUFFER-ELUTED BOVINE DBM

Time (hr)	DBM Weight in GuHCl (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
0	0.299	799.448	26737.390	26.737
0	0.299	726.470	24296.639	24.297
2	0.300	599.793	19993.095	19.993
2	0.300	865.618	28853.938	28.854
4	0.302	448.957	14866.112	14.866
4	0.302	462.119	15301.968	15.302
4	0.299	183.694	6143.601	6.144
4	0.299	186.099	6224.060	6.224
6	0.300	577.573	19252.444	19.252
6	0.300	448.543	14951.432	14.951
6	0.303	456.832	15076.952	15.077
6	0.303	142.282	4695.779	4.696
8	0.299	693.434	23191.769	23.192
8	0.299	830.846	27787.502	27.788
8	0.301	522.164	17347.647	17.348
8	0.301	416.355	13832.389	13.832
24	0.300	610.124	20337.467	20.337
24	0.300	596.865	19895.506	19.896
72	0.298	405.146	13595.493	13.595
72	0.298	392.600	13174.487	13.174
72	0.340	706.068	20766.719	20.767
72	0.340	319.623	9400.662	9.401
168	0.304	810.593	26664.245	26.664
168	0.304	879.860	28942.766	28.943
168	0.300	705.288	23509.604	23.510
168	0.300	716.976	23899.209	23.899
336	0.298	775.525	26024.319	26.024
336	0.298	640.009	21476.821	21.477
336	0.304	678.156	22307.769	22.308
336	0.304	789.634	25974.802	25.975
504	0.301	558.865	18566.951	18.567
504	0.301	481.078	15982.653	15.983
504	0.305	608.156	19939.545	19.940
504	0.305	555.078	18199.267	18.199
672	0.300	348.583	11619.428	11.619
672	0.300	497.571	16585.703	16.586

APPENDIX C (Continued)

TABLE XVIII: GuHCl RUN 1: [BMP-7] IN BUFFER

Time (hr)	DBM Weight in SB (g)	ELISA Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/40ml)
2	1.503	457.484	304.381	12.175
2	1.503	489.516	325.693	13.028
2	1.500	342.657	228.438	9.138
2	1.500	383.499	255.666	10.227
4	1.500	546.153	364.102	14.564
4	1.500	365.545	243.697	9.748
4	1.504	526.115	349.811	13.992
4	1.504	444.117	295.290	11.812
6	1.498	368.980	246.315	9.853
6	1.498	324.339	216.515	8.661
6	1.497	136.880	91.436	3.657
6	1.497	383.384	256.102	10.244
8	1.496	627.106	419.189	16.768
8	1.496	517.178	345.707	13.828
8	1.502	221.028	147.156	5.886
8	1.502	150.537	100.225	4.009
24	1.504	622.458	413.868	16.555
24	1.504	339.581	225.785	9.031
24	1.509	87.759	58.157	2.326
24	1.509	233.398	154.671	6.187
72	1.502	280.916	187.028	7.481
72	1.502	132.268	88.061	3.522
72	1.508	645.397	427.982	17.119
72	1.508	810.982	537.786	21.511
168	1.502	324.680	216.165	8.647
168	1.502	344.935	229.650	9.186
168	1.505	349.666	232.336	9.293
168	1.505	946.426	628.854	25.154
336	1.502	369.610	246.079	9.843
336	1.502	342.941	228.323	9.133
336	1.507	643.171	426.789	17.072
336	1.507	366.575	243.248	9.730
504	1.504	439.994	292.549	11.702
504	1.504	535.727	356.201	14.248

APPENDIX C (Continued)

Time (hr)	DBM Weight in SB (g)	ELISA Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/40ml)
504	1.500	1795.299	1196.866	47.875
504	1.500	460.930	307.287	12.291
672	1.500	637.525	425.017	17.001
672	1.500	350.807	233.871	9.355
672	1.505	233.283	155.005	6.200
672	1.505	2197.435	1460.090	58.404

APPENDIX C (Continued)

TABLE XIX: GuHCl RUN 2: [BMP-7] IN BUFFER-ELUTED BOVINE DBM

Time (hr)	Bone Weight in GuHCl (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
0	0.305	1046.791	34321.016	34.321
0	0.305	2925.492	95917.770	95.918
0	0.314	2728.192	86885.096	86.885
0	0.314	2879.273	91696.592	91.697
0	0.298	2110.766	70831.074	70.831
0	0.298	2109.987	70804.933	70.805
0	0.301	2331.531	77459.502	77.460
0	0.301	2261.139	75120.897	75.121
2	0.295	2727.800	92467.797	92.468
2	0.295	2043.779	69280.644	69.281
2	0.298	1987.469	66693.591	66.694
2	0.298	2062.987	69227.752	69.228
2	0.299	2877.125	96224.916	96.225
2	0.299	2391.823	79994.080	79.994
2	0.299	1280.135	42813.880	42.814
2	0.299	2109.208	70542.074	70.542
4	0.305	2284.717	74908.754	74.909
4	0.305	2110.766	69205.443	69.205
4	0.300	1621.946	54064.867	54.065
4	0.300	1981.064	66035.467	66.035
4	0.300	2138.059	71268.633	71.269
4	0.300	2628.650	87621.667	87.622
4	0.301	2625.707	87232.791	87.233
4	0.301	810.304	26920.399	26.920
6	0.305	1580.268	51812.066	51.812
6	0.305	1468.648	48152.393	48.152
6	0.300	1626.280	54209.333	54.209
6	0.300	1543.981	51466.033	51.466
6	0.298	1518.307	50949.899	50.950
6	0.298	1721.051	57753.389	57.753
6	0.298	2094.487	70284.799	70.285
6	0.298	2212.027	74229.094	74.229
8	0.307	1995.101	64987.003	64.987

APPENDIX C (Continued)

Time (hr)	Bone Weight in GuHCl (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
8	0.307	1848.146	60200.195	60.200
8	0.303	1582.364	52223.234	52.223
8	0.303	1540.933	50855.875	50.856
8	0.302	1430.608	47371.126	47.371
8	0.302	2080.427	68888.311	68.888
24	0.304	926.914	30490.592	30.491
24	0.304	2162.651	71139.836	71.140
24	0.299	1930.871	64577.625	64.578
24	0.299	2386.468	79814.983	79.815
24	0.303	1579.745	52136.799	52.137
24	0.303	1360.062	44886.535	44.887
24	0.307	1616.728	52662.150	52.662
24	0.307	1975.159	64337.427	64.337
72	0.295	1825.391	61877.661	61.878
72	0.295	2070.038	70170.780	70.171
72	0.301	1967.872	65377.807	65.378
72	0.301	1189.781	39527.608	39.528
72	0.297	1673.262	56338.788	56.339
72	0.297	1825.601	61468.047	61.468
72	0.304	1540.764	50683.026	50.683
72	0.304	955.305	31424.507	31.425
168	0.300	1623.208	54106.933	54.107
168	0.300	2358.128	78604.267	78.604
168	0.303	1741.857	57487.030	57.487
168	0.303	1751.389	57801.617	57.802
168	0.299	1853.519	61990.602	61.991
168	0.299	1887.103	63113.813	63.114
168	0.306	1775.529	58023.824	58.024
168	0.306	2052.726	67082.549	67.083
336	0.307	1127.716	36733.420	36.733
336	0.307	2102.732	68492.899	68.493
336	0.304	1904.440	62646.053	62.646
336	0.304	2439.916	80260.395	80.260
336	0.302	2255.663	74690.828	74.691
336	0.302	2138.590	70814.238	70.814
336	0.298	1239.999	41610.705	41.611

APPENDIX C (Continued)

Time (hr)	Bone Weight in GuHCl (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
336	0.298	615.342	20649.060	20.649
504	0.301	2410.875	80095.515	80.096
504	0.301	2396.563	79620.033	79.620
504	0.294	2409.598	81959.116	81.959
504	0.294	1237.881	42104.796	42.105
504	0.305	1093.544	35853.902	35.854
504	0.305	1186.861	38913.475	38.913
672	0.298	2267.795	76100.503	76.101
672	0.298	1423.046	47753.221	47.753
672	0.304	1545.677	50844.638	50.845
672	0.304	3087.268	101554.868	101.555
672	0.300	1735.147	57838.233	57.838
672	0.300	4068.251	135608.367	135.608
672	0.305	2966.213	97252.885	97.253
672	0.305	2259.696	74088.393	74.088

APPENDIX C (Continued)

TABLE XX: GuHCl RUN 2: [BMP-7] IN BUFFER

Time (hours)	Bone Weight in Buffer	Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/40ml)
2	1.504	530.580	352.779	14.111
2	1.504	224.635	149.358	5.974
2	1.504	522.887	347.664	13.907
2	1.504	535.211	355.858	14.234
2	1.502	531.351	353.762	14.150
2	1.502	548.398	365.112	14.604
2	1.502	535.984	356.847	14.274
2	1.502	510.645	339.977	13.599
4	1.498	659.571	440.301	17.612
4	1.498	660.407	440.859	17.634
4	1.498	619.995	413.882	16.555
4	1.498	637.180	425.354	17.014
4	1.500	516.756	344.504	13.780
4	1.500	575.868	383.912	15.356
4	1.500	266.969	177.979	7.119
4	1.500	197.108	131.405	5.256
6	1.502	200.111	133.230	5.329
6	1.502	667.111	444.148	17.766
6	1.502	623.253	414.949	16.598
6	1.502	634.713	422.579	16.903
6	1.498	654.565	436.959	17.478
6	1.498	652.901	435.848	17.434
6	1.498	637.180	425.354	17.014
6	1.498	371.510	248.004	9.920
8	1.501	721.049	480.379	19.215
8	1.501	222.422	148.183	5.927
8	1.501	657.900	438.308	17.532
8	1.501	641.301	427.249	17.090
8	1.503	600.588	399.593	15.984
8	1.503	691.682	460.201	18.408
8	1.503	594.973	395.857	15.834
8	1.503	627.336	417.389	16.696
24	1.502	254.634	169.530	6.781
24	1.502	919.533	612.206	24.488

APPENDIX C (Continued)

Time (hours)	Bone Weight in Buffer	Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/40ml)
24	1.502	875.523	582.905	23.316
24	1.502	1114.433	741.966	29.679
24	1.502	863.784	575.089	23.004
24	1.502	837.762	557.764	22.311
24	1.502	556.201	370.307	14.812
24	1.502	451.256	300.437	12.017
72	1.503	610.261	406.029	16.241
72	1.503	326.079	216.952	8.678
72	1.503	1449.155	964.175	38.567
72	1.503	726.297	483.232	19.329
72	1.503	1159.444	771.420	30.857
72	1.503	1113.239	740.678	29.627
72	1.503	670.475	446.091	17.844
72	1.503	505.313	336.203	13.448
168	1.502	2322.846	1546.502	61.860
168	1.502	2632.759	1752.836	70.113
168	1.502	1696.678	1129.613	45.185
168	1.502	2120.768	1411.963	56.479
168	1.495	935.931	626.041	25.042
168	1.495	971.516	649.843	25.994
168	1.495	1012.549	677.290	27.092
168	1.495	1405.928	940.420	37.617
336	1.499	399.093	266.239	10.650
336	1.499	545.286	363.767	14.551
336	1.499	12174.880	8122.001	324.880
336	1.499	4773.046	3184.153	127.366
336	1.495	2182.282	1459.720	58.389
336	1.495	1791.783	1198.517	47.941
336	1.495	1990.903	1331.708	53.268
336	1.495	2059.319	1377.471	55.099
504	1.503	4328.178	2879.693	115.188
504	1.503	367.170	244.291	9.772
504	1.503	1351.038	898.894	35.956
504	1.500	7213.583	4809.055	192.362
504	1.500	7605.804	5070.536	202.821
504	1.500	7432.178	4954.785	198.191

APPENDIX C (Continued)

Time (hours)	Bone Weight in Buffer	Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/40ml)
672	1.501	410.037	273.176	10.927
672	1.501	1700.505	1132.915	45.317
672	1.501	2400.118	1599.013	63.961
672	1.497	12523.890	8365.992	334.640
672	1.497	10119.060	6759.559	270.382

APPENDIX C (Continued)

TABLE XXI: GuHCl RUN 3: [BMP-7] IN BUFFER-ELUTED BOVINE DBM

Time (hr)	DBM Weight in GuHCl (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
0	0.305	680.743	22319.443	22.319
0	0.305	739.887	24258.590	24.259
0	0.313	813.937	26004.377	26.004
0	0.313	756.779	24178.243	24.178
2	0.297	875.787	29487.778	29.488
2	0.297	721.857	24304.949	24.305
2	0.290	668.511	23052.103	23.052
2	0.290	641.128	22107.862	22.108
4	0.309	652.635	21120.874	21.121
4	0.309	710.827	23004.110	23.004
4	0.314	726.116	23124.713	23.125
4	0.314	666.934	21239.936	21.240
6	0.291	671.666	23081.306	23.081
6	0.291	690.948	23743.918	23.744
6	0.310	603.071	19453.903	19.454
6	0.310	493.066	15905.355	15.905
8	0.310	603.475	19466.935	19.467
8	0.310	634.579	20470.290	20.470
8	0.320	775.037	24219.906	24.220
8	0.320	695.648	21739.000	21.739
24	0.284	418.17	14724.296	14.724
24	0.284	400.526	14103.028	14.103
24	0.312	615.69	19733.654	19.734
24	0.312	557.101	17855.801	17.856
72	0.314	573.636	18268.662	18.269
72	0.314	520.879	16588.503	16.589
168	0.311	491.049	15789.357	15.789
168	0.311	445.208	14315.370	14.315
168	0.320	480.599	15018.719	15.019
168	0.320	364.004	11375.125	11.375
336	0.320	335.738	10491.813	10.492
336	0.320	299.826	9369.563	9.370
336	0.318	524.246	16485.723	16.486
336	0.318	496.403	15610.157	15.610

APPENDIX C (Continued)

Time (hr)	DBM Weight in GuHCl (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
504	0.300	539.405	17980.167	17.980
504	0.300	417.79	13926.333	13.926
504	0.316	333.32	10548.101	10.548
504	0.316	330.978	10473.987	10.474
672	0.293	518.532	17697.338	17.697
672	0.293	411.7	14051.195	14.051
672	0.299	508.384	17002.809	17.003
672	0.299	330.298	11046.756	11.047
1008	0.320	437.325	13666.406	13.666
1008	0.320	419.008	13094.000	13.094
1008	0.298	345.862	11606.107	11.606
1008	0.298	262.164	8797.450	8.797
1344	0.308	571.883	18567.630	18.568
1344	0.308	390.432	12676.364	12.676
1344	0.315	390.736	12404.317	12.404
1344	0.315	390.736	12404.317	12.404
2016	0.310	486.943	15707.839	15.708
2016	0.310	466.019	15032.871	15.033
2016	0.314	431.823	13752.325	13.752
2016	0.314	340.195	10834.236	10.834

APPENDIX C (Continued)

TABLE XXII: GuHCl RUN 3: [BMP-7] IN BUFFER

Time (hr)	DBM Weight in SB (g)	ELISA Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/ml)
2	1.497	363.475	242.802	9.712
2	1.497	324.479	216.753	8.670
2	1.503	301.492	200.593	8.024
2	1.503	237.525	158.034	6.321
4	1.498	377.172	251.784	10.071
4	1.498	337.400	225.234	9.009
4	1.503	298.615	198.679	7.947
4	1.503	278.294	185.159	7.406
6	1.503	405.540	269.820	10.793
6	1.503	366.576	243.896	9.756
6	1.502	416.723	277.445	11.098
6	1.502	380.049	253.029	10.121
8	1.501	395.288	263.350	10.534
8	1.501	350.925	233.794	9.352
8	1.501	364.761	243.012	9.720
8	1.501	206.729	137.728	5.509
24	1.504	506.280	336.622	13.465
24	1.504	498.267	331.295	13.252
24	1.504	538.618	358.124	14.325
24	1.504	541.138	359.799	14.392
72	1.499	1043.790	696.324	27.853
72	1.499	1053.419	702.748	28.110
72	1.500	873.487	582.325	23.293
72	1.500	240.213	160.142	6.406
168	1.500	2343.338	1562.225	62.489
168	1.500	1923.430	1282.287	51.291
168	1.501	1963.192	1307.923	52.317
168	1.501	2107.275	1403.914	56.157
336	1.499	2394.133	1597.153	63.886
336	1.499	2618.238	1746.656	69.866
336	1.492	2264.224	1517.576	60.703
336	1.492	1960.760	1314.182	52.567
504	1.506	3545.542	2354.278	94.171
504	1.506	4454.780	2958.021	118.321

APPENDIX C (Continued)

Time (hr)	DBM Weight in SB (g)	ELISA Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/ml)
504	1.497	3809.937	2545.048	101.802
504	1.497	4035.144	2695.487	107.819
672	1.499	3811.506	2542.699	101.708
672	1.499	3676.669	2452.748	98.110
672	1.501	3652.610	2433.451	97.338
672	1.501	3359.147	2237.939	89.518
1008	1.504	4648.720	3090.904	123.636
1008	1.504	4484.417	2981.660	119.266
1008	1.498	3783.803	2525.903	101.036
1008	1.498	4048.867	2702.848	108.114
1344	1.498	4724.124	3153.621	126.145
1344	1.498	4621.017	3084.791	123.392
1344	1.509	4279.128	2835.738	113.430
1344	1.509	4325.450	2866.435	114.657
2016	1.506	4811.175	3194.671	127.787
2016	1.506	4917.907	3265.542	130.622
2016	1.506	4737.347	3145.649	125.826
2016	1.506	7577.732	5031.695	201.268

APPENDIX D

TABLE XXIII: COLLAGENASE DIGESTION: [BMP-7] IN BUFFER-ELUTED BOVINE DBM

Time (hr)	DBM Weight in Collagenase (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
0	0.999	1030.024	6701.858	6.702
0	0.999	1540.225	10021.484	10.021
0	1.017	1535.334	9812.853	9.813
0	1.017	1642.795	10499.673	10.500
2	1.005	1441.480	9323.005	9.323
2	1.005	1372.406	8876.258	8.876
2	1.033	1007.398	6338.903	6.339
2	1.033	1096.062	6896.808	6.897
4	0.997	986.796	6433.474	6.433
4	0.997	1119.698	7299.937	7.300
4	1.000	1236.586	8037.809	8.038
4	1.000	1411.707	9176.096	9.176
6	1.003	1149.569	7449.849	7.450
6	1.003	992.508	6432.006	6.432
6	1.001	1169.776	7595.948	7.596
6	1.001	1107.288	7190.182	7.190
8	0.999	155.326	1010.630	1.011
8	0.999	165.264	1075.291	1.075
8	1.012	1542.254	9905.782	9.906
8	1.012	1428.125	9172.740	9.173
24	1.004	1160.312	7511.980	7.512
24	1.004	1027.686	6653.346	6.653
24	1.001	227.582	1477.805	1.478
24	1.001	294.625	1913.149	1.913
72	1.002	798.123	5177.445	5.177
72	1.002	1184.364	7683.000	7.683
72	1.025	1186.516	7524.248	7.524
72	1.025	1244.006	7888.819	7.889
168	1.012	1253.344	8050.134	8.050
168	1.012	1211.099	7778.798	7.779
168	1.019	349.556	2229.749	2.230
168	1.019	1546.153	9862.605	9.863
336	1.014	1018.824	6530.923	6.531
336	1.014	1008.492	6464.692	6.465
336	0.999	1153.756	7506.921	7.507
336	0.999	1368.426	8903.673	8.904
504	0.580	842.924	9446.562	9.447
504	0.580	1152.031	12910.692	12.911
504	0.747	1070.918	9318.564	9.319
504	0.747	448.364	3901.427	3.901
672	1.005	881.434	5700.817	5.701
672	1.005	1070.688	6924.848	6.925
672	1.000	1234.739	8025.804	8.026

APPENDIX D (Continued)

Time (hr)	DBM Weight in Collagenase (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
672	1.000	1239.761	8058.447	8.058
1008	1.000	820.858	5335.577	5.336
1008	1.000	771.877	5017.201	5.017
1008	0.996	837.470	5465.417	5.465
1008	0.996	858.267	5601.140	5.601
1344	1.020	805.060	5130.284	5.130
1344	1.020	761.299	4851.415	4.851
1344	1.000	662.213	4304.385	4.304
1344	1.000	688.972	4478.318	4.478
2016	1.000	687.182	4466.683	4.467
2016	1.000	624.156	4057.014	4.057
2016	0.990	642.209	4216.524	4.217
2016	0.990	766.177	5030.455	5.030

APPENDIX D (Continued)

TABLE XXIV: COLLAGENASE DIGESTION: [BMP-7] IN BUFFER

Time (hr)	DBM Weight in SB (g)	ELISA Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/40ml)
2	1.503	209.674	139.504	5.580
2	1.503	286.047	190.317	7.613
2	1.504	288.426	191.773	7.671
2	1.504	243.560	161.941	6.478
4	1.503	277.240	184.458	7.378
4	1.503	135.642	90.248	3.610
4	1.504	320.034	212.789	8.512
4	1.504	271.890	180.778	7.231
6	1.503	285.974	190.269	7.611
6	1.503	365.219	242.993	9.720
6	1.498	338.964	226.278	9.051
6	1.498	318.236	212.441	8.498
8	1.499	297.146	198.229	7.929
8	1.499	351.719	234.636	9.385
8	1.501	396.107	263.895	10.556
8	1.501	396.398	264.089	10.564
24	1.499	465.765	310.717	12.429
24	1.499	457.495	305.200	12.208
24	1.500	373.609	249.073	9.963
24	1.500	404.414	269.609	10.784
72	1.500	600.347	400.231	16.009
72	1.500	593.292	395.528	15.821
72	1.500	670.981	447.321	17.893
72	1.500	599.785	399.857	15.994
168	1.495	869.933	581.895	23.276
168	1.495	1054.327	705.235	28.209
168	1.501	910.202	606.397	24.256
168	1.501	899.036	598.958	23.958
336	1.500	132.377	88.251	3.530
336	1.500	195.076	130.051	5.202
336	1.498	1324.771	884.360	35.374
336	1.498	1510.334	1008.234	40.329
504	1.499	2127.063	1418.988	56.760
504	1.499	2011.074	1341.610	53.664
504	1.502	2085.106	1388.220	55.529
504	1.502	2115.543	1408.484	56.339
672	1.500	830.722	553.815	22.153
672	1.500	255.070	170.047	6.802
672	1.504	292.391	194.409	7.776
672	1.504	1910.152	1270.048	50.802
1008	1.500	3350.340	2233.560	89.342
1008	1.500	3422.385	2281.590	91.264
1008	1.500	3549.426	2366.284	94.651
1008	1.500	3657.034	2438.023	97.521

APPENDIX D (Continued)

Time (hr)	DBM Weight in SB (g)	ELISA Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/40ml)
1344	1.510	3802.890	2518.470	100.739
1344	1.510	3617.131	2395.451	95.818
1344	1.500	3432.807	2288.538	91.542
1344	1.500	3388.408	2258.939	90.358
2016	1.490	3477.347	2333.790	93.352
2016	1.490	3526.913	2367.056	94.682
2016	1.500	3418.028	2278.685	91.147
2016	1.500	3909.473	2606.315	104.253

APPENDIX E

TABLE XXV: [BMP-2] IN BUFFER-ELUTED HUMAN DBM

Time (hr)	Bone Weight in GuHCl (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
0	0.309	837.515	27104.045	27.104
0	0.309	927.442	30014.304	30.014
0	0.315	867.475	27538.889	27.539
0	0.315	868.280	27564.444	27.564
2	0.287	1112.220	38753.310	38.753
2	0.287	1142.119	39795.087	39.795
2	0.289	947.755	32794.291	32.794
2	0.289	979.076	33878.062	33.878
4	0.300	1016.476	33882.533	33.883
4	0.300	1172.806	39093.533	39.094
4	0.295	1086.087	36816.508	36.817
4	0.295	1232.600	41783.051	41.783
6	0.309	1220.588	39501.230	39.501
6	0.309	1094.210	35411.327	35.411
6	0.303	1114.418	36779.472	36.779
6	0.303	1113.685	36755.281	36.755
8	0.292	955.912	32736.712	32.737
8	0.292	1041.000	35650.685	35.651
8	0.318	1097.158	34501.824	34.502
8	0.318	1016.476	31964.654	31.965
24	0.312	1156.236	37058.846	37.059
24	0.312	1126.842	36116.731	36.117
24	0.298	1165.253	39102.450	39.102
24	0.298	1154.069	38727.148	38.727
72	0.298	995.169	33394.933	33.395
72	0.298	1102.677	37002.584	37.003
72	0.305	960.562	31493.836	31.494
72	0.305	1102.677	36153.344	36.153
168	0.302	868.280	28750.993	28.751
168	0.302	885.505	29321.358	29.321
168	0.296	830.575	28059.966	28.060
168	0.296	824.434	27852.500	27.853

APPENDIX E (Continued)

TABLE XXVI: HUMAN [BMP-2] IN BUFFER

Time (hours)	Bone Weight in Buffer (g)	ELISA Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/40ml)
2	1.787	79.987	44.760	1.790
2	1.787	66.557	37.245	1.490
2	1.678	42.196	25.147	1.006
2	1.678	49.610	29.565	1.183
4	1.693	20.665	12.206	0.488
4	1.693	31.430	18.565	0.743
4	1.764	53.160	30.136	1.205
4	1.764	51.987	29.471	1.179
6	1.850	77.980	42.151	1.686
6	1.850	83.944	45.375	1.815
6	1.859	73.905	39.755	1.590
6	1.859	65.483	35.225	1.409
8	1.766	75.953	43.008	1.720
8	1.766	51.987	29.438	1.178
8	1.823	73.905	40.540	1.622
8	1.823	67.626	37.096	1.484
24	1.839	98.198	53.397	2.136
24	1.839	118.645	64.516	2.581
24	1.686	76.969	45.652	1.826
24	1.686	85.895	50.946	2.038
72	1.941	113.450	58.449	2.338
72	1.941	130.443	67.204	2.688
72	1.810	134.558	74.341	2.974
72	1.810	122.060	67.436	2.697
168	1.777	142.647	80.274	3.211
168	1.777	137.004	77.098	3.084
168	1.714	122.908	71.708	2.868
168	1.714	143.446	83.691	3.348

APPENDIX E (Continued)

TABLE XXVII: [BMP-4] IN BUFFER-ELUTED HUMAN DBM

Time (hr)	Bone Weight in GuHCl (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
0	0.309	16.115	521.521	0.522
0	0.309	17.442	564.466	0.564
0	0.315	20.660	655.873	0.656
0	0.315	17.868	567.238	0.567
2	0.287	26.675	929.443	0.929
2	0.287	25.902	902.509	0.903
2	0.289	24.911	861.972	0.862
2	0.289	24.088	833.495	0.833
4	0.300	36.217	1207.233	1.207
4	0.300	31.749	1058.300	1.058
4	0.295	30.180	1023.051	1.023
4	0.295	30.627	1038.203	1.038
6	0.309	31.749	1027.476	1.027
6	0.309	31.693	1025.663	1.026
6	0.303	25.296	834.851	0.835
6	0.303	26.454	873.069	0.873
8	0.292	20.444	700.137	0.700
8	0.292	24.143	826.815	0.827
8	0.318	29.900	940.252	0.940
8	0.318	32.030	1007.233	1.007
24	0.312	28.228	904.744	0.905
24	0.312	31.188	999.615	1.000
24	0.298	23.541	789.966	0.790
24	0.298	27.229	913.725	0.914
72	0.298	20.012	671.544	0.672
72	0.298	24.417	819.362	0.819
72	0.305	19.905	652.623	0.653
72	0.305	20.552	673.836	0.674
168	0.302	17.761	588.113	0.588
168	0.302	18.723	619.967	0.620
168	0.296	15.219	514.155	0.514
168	0.296	14.380	485.811	0.486

APPENDIX E (Continued)

TABLE XXVIII: HUMAN [BMP-4] IN BUFFER

Time (hr)	Bone Weight in buffer (g)	ELISA Result (pg/ml)	Adjusted Result: 0.5 Dilution (pg/ml)	Normalized Adjusted Result (pg/g.ml)	Normalized Adjusted Result (ng/g/40ml)
2	1.787	0.000	0.000	0.000	0.00000
2	1.787	0.000	0.000	0.000	0.00000
4	1.693	0.000	0.000	0.000	0.00000
4	1.693	0.000	0.000	0.000	0.00000
6	1.850	0.072	0.036	0.019	0.00078
6	1.850	0.072	0.036	0.019	0.00078
8	1.766	0.000	0.000	0.000	0.00000
8	1.766	0.000	0.000	0.000	0.00000
24	1.839	0.365	0.183	0.099	0.00397
24	1.839	0.681	0.341	0.185	0.00741
24	1.686	0.000	0.000	0.000	0.00000
24	1.686	0.039	0.020	0.012	0.00046
72	1.941	2.181	1.091	0.562	0.02247
72	1.941	2.585	1.293	0.666	0.02664
72	1.810	1.137	0.569	0.314	0.01256
72	1.810	1.435	0.718	0.396	0.01586
168	1.777	2.047	1.024	0.576	0.02304
168	1.777	2.003	1.002	0.564	0.02254
168	1.714	1.827	0.914	0.533	0.02132
168	1.714	2.047	1.024	0.597	0.02389

APPENDIX E (Continued)

TABLE XXIX: [BMP-7] IN BUFFER-ELUTED HUMAN DBM

Time (hr)	Bone Weight in GuHCl (g)	ELISA Result (pg/ml)	Normalized Result (pg/g)	Normalized Result (ng/g)
0	0.309	2809.742	90930.162	90.930
0	0.309	2786.695	90184.304	90.184
0	0.315	2727.671	86592.730	86.593
0	0.315	3266.487	103698.000	103.698
2	0.287	2866.886	99891.498	99.891
2	0.287	3125.110	108888.850	108.889
2	0.289	2735.189	94643.218	94.643
2	0.289	2905.088	100522.076	100.522
4	0.300	3054.238	101807.933	101.808
4	0.300	3049.998	101666.600	101.667
4	0.295	2967.451	100591.559	100.592
4	0.295	3082.031	104475.627	104.476
6	0.309	2875.142	93046.667	93.047
6	0.309	3380.841	109412.330	109.412
6	0.303	2797.473	92325.842	92.326
6	0.303	3605.752	119001.716	119.002
8	0.292	3094.231	105966.815	105.967
8	0.292	3305.863	113214.486	113.214
8	0.318	3055.606	96088.239	96.088
8	0.318	3531.069	111039.906	111.040
24	0.312	3394.648	108802.821	108.803
24	0.312	3276.738	105023.654	105.024
24	0.298	2842.005	95369.295	95.370
24	0.298	2848.087	95573.389	95.573
72	0.298	3024.577	101495.872	101.496
72	0.298	2948.677	98948.893	98.949
72	0.305	2987.608	97954.361	97.954
72	0.305	3276.600	107429.508	107.430
168	0.302	2836.466	93922.715	93.923
168	0.302	3166.783	104860.364	104.860
168	0.296	2667.380	90114.189	90.114
168	0.296	3331.561	112552.736	112.553

APPENDIX E (Continued)

TABLE XXX: HUMAN [BMP-7] IN BUFFER

Time (hr)	Bone Weight in Buffer (g)	ELISA Result (pg/ml)	Normalized Result (pg/g.ml)	Normalized Result (ng/g/40ml)
2	1.787	538.959	301.600	12.064
2	1.787	540.952	302.715	12.109
2	1.678	493.321	293.993	11.760
2	1.678	505.711	301.377	12.055
4	1.693	511.910	302.369	12.095
4	1.693	495.307	292.562	11.702
4	1.764	534.272	302.875	12.115
4	1.764	547.987	310.650	12.426
6	1.850	547.166	295.765	11.831
6	1.850	577.452	312.136	12.485
6	1.859	500.567	269.267	10.771
6	1.859	539.194	290.045	11.602
8	1.766	567.467	321.329	12.853
8	1.766	570.991	323.324	12.933
8	1.823	597.206	327.595	13.104
8	1.823	601.090	329.726	13.189
24	1.839	702.649	382.082	15.283
24	1.839	804.655	437.550	17.502
24	1.686	590.736	350.377	14.015
24	1.686	599.207	355.402	14.216
72	1.941	782.008	402.889	16.116
72	1.941	794.996	409.581	16.383
72	1.810	798.931	441.398	17.656
72	1.810	866.450	478.702	19.148
168	1.777	1374.497	773.493	30.940
168	1.777	1222.224	687.802	27.512
168	1.714	1066.057	621.970	24.879
168	1.714	1092.767	637.554	25.502

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