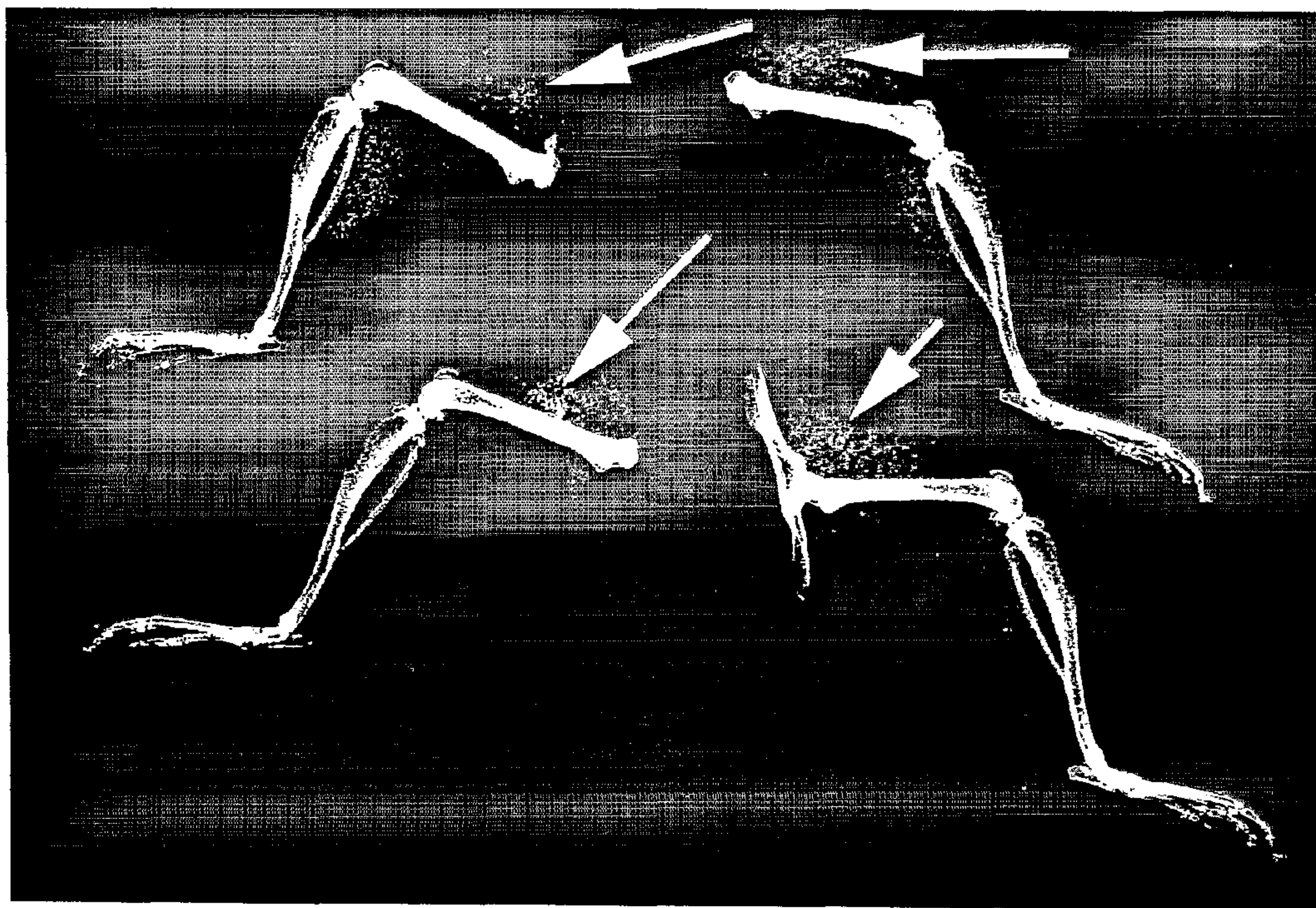




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THE REGENTS OF THE UNIVERSITY OF CALIFORNIA,
US;
DEPARTMENT OF VETERANS AFFAIRS, US
(72) Inventeurs/Inventors:
BEHNAM, KEYVAN, US;
MURRAY, ELSA J., US;
MURRAY, SAMUEL S., US
(74) Agent: MCCARTHY TETRAULT LLP

(54) Titre : ACTIVATION DE L'EXTRACTION DE LA MATRICE OSSEUSE DEMINERALISEE
(54) Title: ACTIVATING EXTRACTION OF DEMINERALIZED BONE MATRIX



DMB

Extracted DMB

(57) Abrégé/Abstract:

Demineralized bone matrix (DBM) and native bone morphogenetic protein (nBMP) are complex mixtures of non-collagenous bone proteins. These mixtures contain many of the BMPs that are available as recombinant molecules. Information regarding the presence in these materials of molecules that may affect the availability and activity of the BMPs is very limited. A chemical extraction of DBM, such as using alkali-urea produces a water soluble extractate which inhibits the osteogenic activity of DBM. Noggin, an extracellular BMP ligand antagonist is found in the water soluble extractate from DBM. Differential chemical extraction is a useful means of removing non-osteogenic, or osteogenic inhibitory molecules from DBM and nBMP.

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(71) Applicants (for all designated States except US): **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 4th Floor, Oakland, California 94607-5200 (US). **DEPARTMENT OF VETERANS AFFAIRS** [US/US]; Office Of The General Counsel, Washington, District Of Columbia 20420 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BEHNAM, Keyvan** [US/US]; Red Bank, New Jersey (US). **MURRAY, Elsa, J.** [US/US]; Saugus, California (US). **MURRAY, Samuel, S.** [US/US]; Saugus, California (US).(74) Agent: **BISBIKIS, John, G.**; MCDERMOTT WILL & EMERY LLP, 227 West Monroe Street, Chicago, Illinois 60606 (US).

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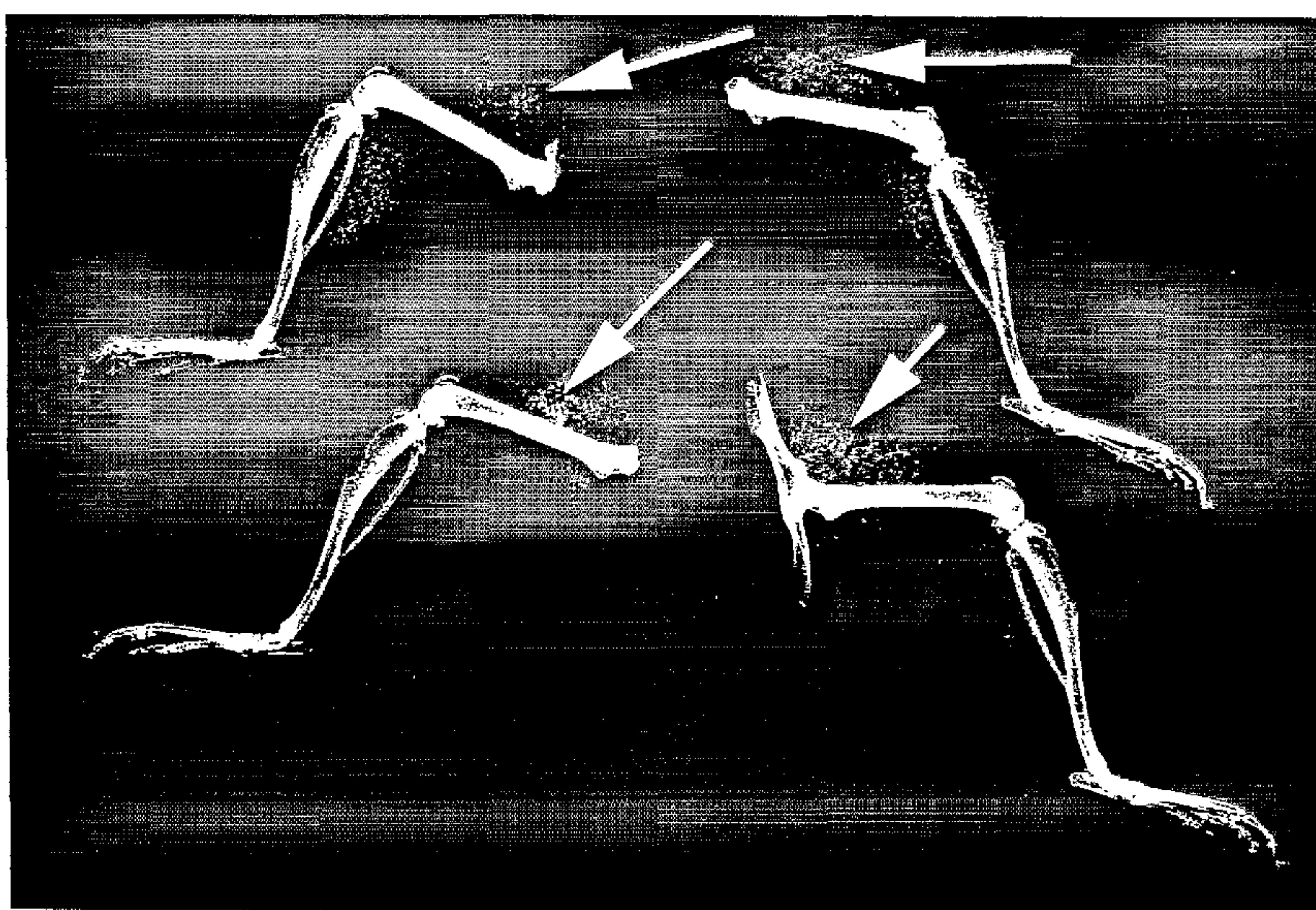
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(54) Title: ACTIVATING EXTRACTION OF DEMINERALIZED BONE MATRIX



DMB

Extracted DMB

(57) Abstract: Demineralized bone matrix (DBM) and native bone morphogenetic protein (nBMP) are complex mixtures of non-collagenous bone proteins. These mixtures contain many of the BMPs that are available as recombinant molecules. Information regarding the presence in these materials of molecules that may affect the availability and activity of the BMPs is very limited. A chemical extraction of DBM, such as using alkali-urea produces a water soluble extractate which inhibits the osteogenic activity of DBM. Noggin, an extracellular BMP ligand antagonist is found in the water soluble extractate from DBM. Differential chemical extraction is a useful means of removing non-osteogenic, or osteogenic inhibitory molecules from DBM and nBMP.

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ACTIVATING EXTRACTION OF DEMINERALIZED BONE MATRIX

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0001] This work was supported by the Department of Veterans' Affairs. The United States government may have certain rights in the invention.

FIELD OF THE INVENTION

[0002] The invention generally relates to osteogenic factors, and more particularly to demineralized bone matrix ("DBM") and/or native bone morphogenetic protein ("nBMP"), to compositions including DBM and/or nBMP having at least one endogenous inhibitor of osteogenic activity removed therefrom, articles of manufacture including DBM and/or nBMP and methods of using the compositions and articles to induce osteogenesis.

BACKGROUND OF THE INVENTION

[0003] Normal bone formation occurs during development and repair after injury and remodeling occurs in adult life to preserve the integrity of the skeleton. Bone formation and remodeling generally involve bone resorption by osteoclasts and bone formation by osteoblasts, which are regulated by growth factors. Thus, any interference between the balance in cell differentiation, bone formation and bone resorption can affect bone formation, repair and homeostasis.

[0004] Noggin is a protein which is present and highly conserved in both protostome and deuterostome phyla and which interacts with bone morphogenetic proteins ("BMPs") to affect embryonic orientation and tissue layer formation in early development. (Reddi, A.H. (2001). Interplay between bone morphogenetic proteins and cognate binding proteins in bone and cartilage development: noggin, chordin and DAN. *Arthritis Res.* 3:1-5. Zimmerman; L.B., et al. (1996) The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86:599-606.) It is a 33 kD glycoprotein which is secreted as a 64 kD homodimer and was shown to perform the functions of the active principle of the Spemann organizer in *Xenopus* in that it induced neural tissue from dorsal ectoderm and changed the specification of lateral mesoderm from ventral (blood, mesenchyme) to dorsal (muscle, heart, pronephros) fates during gastrulation (Smith, W.C., et al. (1993). Secreted noggin protein mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm. *Nature* 361:547-549. Lamb, T.M., et al. (1993) Neural induction by the secreted polypeptide noggin.

Science 262:713-718). BMP-4 had been demonstrated to act as a ventralizing factor in *Xenopus* development (Dale, L., et al. (1992) Bone morphogenetic protein 4: a ventralizing factor in early *Xenopus* development. Development 115:573-585. Jones, C.M., et al. (1992) DVR-4 (bone morphogenetic protein-4) as a posterior-ventralizing factor in *Xenopus* mesoderm induction. Development 115:639-647) and to inhibit neural induction (Wilson, P.A., and Hemmati-Brivanlou, A. (1995) Induction of epidermis and inhibition of neural fate by BMP 4. Nature 376:331-333) suggesting that noggin may function by interacting with BMP-4. Subsequent studies demonstrated that noggin avidly binds BMP-4 and inhibits its activity by blocking binding of BMP-4 to cellular receptors (Zimmerman, L.B., et al. (1996) The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell 86:599-606. Groppe, J., Greenwald, J., et al. (2002) Structural basis of BMP signaling inhibition by the cystein knot protein noggin. Nature 420:636-642).

[0005] Noggin performs important regulatory functions in the vertebrate skeleton (Brunet, L.J., et al. (1998) Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. Science 280:1455-1457. Devlin, R.D., et al. (2003) Skeletal over expression of noggin results in osteopenia and reduced bone formation. Endocrinology 144:1972-1978. Nakamura, Y., Wakitani, S., Nakayama, J., et al. (2003) Temporal and spatial expression profiles of BMP receptors and noggin during bmp-2-induced ectopic bone formation. J. Bone Min. Res. 18:1854-1862. Stottmann, R.W., et al. (2001) The BMP antagonists chordin and noggin have essential but redundant roles in mouse mandibular outgrowth. Dev. Biol. 240:457-473). Noggin reduced BMP-4-induced expression of alkaline phosphatase in W-20-17 mouse bone marrow stromal cells, which were isolated on the basis of manifesting an osteoblast-like phenotype after treatment with rhBMP-2 (Theis, R.S., et al. (1992) Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology 130:1318-1324). Noggin was shown to be expressed in condensing cartilage in mice, and mice genetically altered to lack noggin were found to have shorter but thicker limbs and multiple synarthroses (Brunet, L.J., et al. (1998) Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. Science 280:1455-1457). Human mutations of the noggin gene have been shown to be associated with *fibrodysplasia ossificans*

progressiva and synarthrosis syndromes (Semonin, O., et al. (2001)

Identification of three novel mutations of the noggin gene in patients with fibrodysplasia ossificans progressiva. Am. J. Med. Genet. 102:314-317. Gong, Y., et al. (1999) Heterozygous mutations in the gene encoding noggin affect human joint morphogenesis. Nature Genet. 21:302-304).

[0006] Delivery of modified or wild-type noggin in a bone chamber reduced in-growth of new bone at four weeks in rats (Aspenberg, P., et al. (2001) The bone morphogenetic proteins antagonist noggin inhibits membranous ossification. J. Bone Mineral. Res. 16:497-500). Transgenic mice which over-expressed noggin under the control of the osteocalcin promoter exhibited diminished bone mineral density, reduced trabecular number and volume, reduced bone formation rate, but normal trabecular osteoclast surface with reduced osteoclast number (Devlin, R.D., et al. (2003) Skeletal over expression of noggin results in osteopenia and reduced bone formation. Endocrinology 144:1972-1978). Noggin delivered through a retroviral vector inhibited ectopic bone formation induced by demineralized bone matrix or vector-delivered BMP-4 (Hannallah, D., et al. (2004) Retroviral delivery of noggin inhibits the formation of heterotopic ossification induced by BMP-4, demineralized bone matrix, and trauma in an animal model. J. Bone Joint Surg. 86:80-91).

[0007] Demineralized bone matrix (DBM) and native bone morphogenetic protein (nBMP) are partially purified extracts of non-collagenous bone proteins that contain at least one endogenous BMP. BMP's belong to a family of growth factors described as bone morphogenic protein/non-collagenous protein (BMP/NCP or BMPs). Specifically BMP-2 and BMP-4 have been isolated therefrom, and are in the TGF- β family of proteins. However, DBM and nBMP also include enhancers and inhibitors of BMP activity.

[0008] For example, recently BBP has been disclosed as enhancing ossification caused by recombinant BMP. Further, BBP as used with BMP *in vivo* causes osteogenesis to occur faster, to a greater extent and with smaller amounts of rhBMP-2.

[0009] DBM and nBMP exhibit osteogenic potential, and can be used to enhance bone healing in a number of clinical situations. (Urist, M.R. (1965) Bone: Formation by autoinduction. Science 150:893-899; Reddi, A.H. and Huggins, C. (1972) Biochemical sequences in the transformation of normal

fibroblasts in adolescent rats. Proc. Natl. Acad. Sci. U.S.A. 69:1601-31605 and Urist, M.R., et al. (1984) Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography. Proc. Natl. Acad. Sci. U.S.A. 81:371-375. (Urist, M.R. Emerging Concepts of Bone Morphogenetic Protein. (1991) In: Fundamentals of Bone Growth: Methodology and Applications, A.D. Dixon, B.G. Sarnat, and D.A.N. Hoyte (eds.), pp. 189-198. (C.R.C. Press, Boston)). Because of the number of BMP-related proteins found in DBM and the complexity of their regulatory functions, it is likely that partially purified mixtures such as DBM and nBMP contain a number of molecules that affect the availability and activity of the BMPs. While recombinant BMPs are available, the cost of using minimally effective dosages of BMP has been a limiting factor in clinical use. Therefore, DBM and nBMP having increased osteogenic activity are desired.

[0010] Safe, effective and affordable compositions, devices and methods are desired to treat bone disorders (such as osteoporosis), bone injury (such as fracture healing of flat (e.g., membranous) and long (e.g., endochondral) bones, non-union fractures and reconstructive surgery), sites of knee/hip/joint repair or replacement surgery treating periodontitis, periodontal regeneration, alveolar ridge augmentation for tooth implant reconstruction, for example.

[0011] Further, the use of compositions and methods in combination with other methods for enhancing osteogenesis are desirable.

[0012] It may be desirable to improve DBM and nBMP by adding activators and substances which improve the kinetics or overall osteogenic potential of BMP action or by removing inhibitors of BMP action, for example.

[0013] Further, methods for removing inhibitors of BMPs, while not adversely effecting the osteogenic activity of BMPs in DBM (such as BMP-2, -4 and -7 (known as "osteogenin"), are desirable. Also desirable are DBM and/or rBMP which produces more bone per volume or weight than native DBM or nBMP obtained from non-processed DBM. Finally, methods which do not adversely effect the osteogenic potential of DBM are desirable.

SUMMARY OF THE INVENTION

[0014] In one aspect of the invention, it is desirable to increase the osteogenic activity of DBM or nBMP by reducing the levels of or removing inhibitors of osteogenic activity therefrom, and more specifically to remove inhibitors of BMP action therefrom.

[0015] In one aspect of the invention, an alkali-urea extraction may be used to substantially reduce the levels of or remove inhibitory proteins, including but not limited to noggin, in a water soluble extractate from DBM or nBMP.

[0016] The invention may include a method of systemic delivery or localized treatment with agents for maintaining bone homeostasis, enhancing bone formation and/or enhancing bone repair. The invention may include a method of systemic delivery or localized treatment with cells for maintaining bone homeostasis, enhancing bone formation and/or enhancing bone repair.

[0017] In one application of the invention, the method may be applied to induce the formation, local repair, effective bone homeostasis in bone related disorders, such as osteoporosis.

[0018] The invention may also include devices such as implants having coatings of substances or seeded with differentiated cells for inducing bone formation or enhancing bone repair. The invention may also include the application of substances or differentiated cells at a site where bone formation or bone repair is desired. For example, implants may include, but are not limited to pins, screws and plates that are used to immobilize a fracture, enhance bone formation or stabilize a prosthetic implant by stimulating bone formation or bone repair.

[0019] This invention is advantageous at least in that DBM or nBMP, for example may enhance osteogenesis and the activity of other osteogenic materials, such as BMP-2, BMP-4 or BMP-7. Therefore, lower doses of osteogenic materials may be used for clinical applications. This is significant at least in that clinical treatments may be more affordable. Further this invention is advantageous at least in that BBP may be used to further enhance the osteogenic potential of DBM and/or nBMP.

[0020] These, as well as other objects, features and benefits will now become clear from a review of the following detailed description of illustrative embodiments and the accompanying drawings.

BRIEF DESCRIPTION OF DRAWINGS

[0021] FIG. 1 is a radiogram of hindquarters of nude mice 28 days after implantation with 25 mg of either DBM (left) or extracted DBM (right). Ectopic

bone formation is observed in all the specimens (see arrows; note areas of increased opacity above the femur).

[0022] FIG. 2 is a radiogram of hindquarters of nude mice 28 days after implantation with 25 mg of extracted DBM plus 25 mg of the water soluble fraction of the alkali-urea extractate. (Arrows indicate the site of implantation; material in the concentrated extractate has inhibited the osteogenic potential of the DBM.)

[0023] FIG. 3 is a Western blot for the detection of noggin in protein representing 2.5 mg of each test material. Lanes A: Water soluble fraction of the alkali-urea extractate of DBM; B: Alkali-urea extracted DBM; C: Untreated DBM; D: 1 μ g recombinant noggin/Fc chimeric protein. (Noggin is present in the extractate and untreated DBM, but not the treated DBM. The arrow points to the recombinant noggin in lane D with an expected M_r of 64 kD. Native noggin seen in lanes A, B, or C, is expected to have a M_r of 60 kD.)

[0024] FIG. 4 is a flow chart of one method of the invention.

[0025] FIGS. 5 A & B are schematic depictions of two embodiments of the present invention.

[0026] FIG. 6 is a flow chart of one method of the invention.

DETAILED DESCRIPTION

[0027] Demineralized bone matrix (DBM) and the native bone morphogenetic protein (nBMP) that is purified from it induce ectopic endochondral bone formation that can be used to enhance bone healing in a number of clinical situations. These materials are complex mixtures of numerous proteins and contain a number of the known bone morphogenetic proteins (BMPs) and, possibly, BMP-binding proteins or inhibitors of BMPs. Therefore, DBM-related materials with increased osteogenic activity are desired.

[0028] The present invention is related to agents such as DBM, nBMP and/or proteins and methods for maintaining bone homeostasis, enhancing osteogenesis to promote bone formation and/or enhancing bone repair.

[0029] One embodiment may include a method for making and a composition comprising DBM and/or nBMP extracted to remove or reduce the amount of non-osteogenic proteins (such as noggin). Extraction may be with a chaotropic solvent, such as urea in a concentration from about 1M to 4.5M, for example.

Preferably DBM extraction should be undertaken in a way so as to not eliminate the osteoinductive activity of the DBM or nBMP therein.

[0030] Further, one embodiment may include a method for making and a composition comprising proteins, such as non-collagenous proteins from extracted DBM or nBMP as described above. A second extraction may be with one of a chaotropic solvent (for example, about 4M guanidinium HCL, 6M urea), an ionic detergent (for example, about 1% sodium dodecylsulfate) or nondenaturing concentrations of acid (for example, about 1 to about 2M citric acid).

[0031] More specifically, embodiments of the invention may include the systemic and/or local application of agents for maintaining bone homeostasis, enhancing bone formation and/or enhancing bone repair. Clinical indices of a method or compounds ability to maintain bone homeostasis is evidenced by improvements in bone density at different sites through out the body as assessed, at least by DEXA scanning. Enhanced bone formation in a healing fracture is routinely assessed by regular X-ray of the fracture site at selected time intervals. More advanced techniques for determining the above indices, such as quantitative CT scanning may be used.

[0032] More specifically, embodiments of the invention may include the use of agents which stimulate osteogenesis. Embodiments of the invention may include the use of agents which induce the proliferation of precursor cells which subsequently differentiate into bone cells (osteoblasts), such as DBM and/or nBmp.

[0033] Embodiments of the invention may also include the use of agents which inhibit osteoclastic bone resorption. Agents which may be useful in this invention to effect osteoclastic bone resorption include, but are not limited to: bisphosphonates, selective estrogen receptor modulators, calcitonin, vitamin D and/or calcium supplementation. Embodiments of the invention may also include the use of agents which induce osteoblastic bone formation. Agents which may be useful in this invention include, but are not limited to PTH, sodium fluoride and growth factors, such as insulin-like growth factors I and II and transforming growth factor beta.

[0034] The *in vivo* models used to show the osteogenic effects of extracted DBM, nBMP or non-collagenous proteins have been used previously in

demonstrating similar behaviors of other osteogenic compounds. In particular, *in vivo* models have previously been able to successfully predict the *in vivo* osteogenic effects of compounds such as BMP and insulin like growth factors (IGF). Specifically, it has been demonstrated that BBP enhances the osteogenic potential of BMP-2 in a mouse hindquarter assay. Demonstration of osteogenic effects of a compound in these *in vivo* models are necessary prior to trials that would demonstrate their effects *in vivo* humans.

[0035] Agents which may be useful in this invention to effect osteogenesis include, but are not limited to extracted DBM, nBMP and/or non-collagenous proteins alone or in combination with other osteogenic agents, such as BMP-2, BMP-4, BMP-7 or any portion of these peptides which are found to be active in effecting calcification or osteogenesis. Further, BPP may be added to enhance the osteogenic potential of BMP-2, for example.

[0036] Therapeutically effective dose. A therapeutically effective dose of a agent useful in this invention is one which has a positive effect on a patient or desired effect in cells as measured by the ability of the agent to enhance calcification or osteogenesis, as described above. The therapeutically effective dose of each agent can be modulated to achieve the desired clinical effect, while minimizing negative side effects. The dosage of the agent may be selected for an individual application depending upon the route of administration, severity of the disease, age and weight of a patient, other medications the patient is taking and other factors normally considered by an attending physician, when determining an individual regimen and dose level appropriate for a particular patient. It is desirable at least that the extracted DBM, nBMP, or non-collagenous proteins may lead to a more active therapeutic material relative to the same weight or volume of native DBM or native BMP ("non-extracted"). The therapeutically effective dosage of DBM and nBMP substantially free of noggin may be determined to be less than that of DBM and nBMP including noggin.

[0037] Dosage Form. The therapeutically effective dose of an agent included in the dosage form may be selected by considering the type of agent selected and the route of administration. The dosage form may include an agent in combination with other inert ingredients, including adjuvants and pharmaceutically acceptable carriers for the facilitation of dosage to the patient, as is known to those skilled in the pharmaceutical arts.

[0038] Therapeutic formulations may be prepared for storage by mixing the agents having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers, in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; anti-oxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins. Other components can include glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

[0039] The dosage form may be provided in a topical preparation (e.g., lotion, crème, ointment, transdermal patch, or the like) for dermal application. The dosage form may also be provided in preparations for subcutaneous (such as in a slow-release capsule), intravenous, intraperitoneal, intramuscular or respiratory application, for example.

[0040] In one embodiment, the dosage form may be an oral preparation (e.g., liquid, capsule, caplet or the like) which when consumed results in the elevated levels of the agent in the body. The oral preparation may comprise carriers including diluents, binders, time release agents, lubricants and disintegrants.

[0041] Any one or a combination of agents may be included in a dosage form. Alternatively, a combination of agents may be administered in separate dosage forms. A combination of agents may be administered concurrent in time such that there is exposure to at least two agents for treatment.

[0042] Additional Agents. The invention may include treatment with an additional agent which acts independently or synergistically with extracted DBM, nBMP and/or DMP proteins to enhance osteogenesis and/or calcification. For example, DBM and/or nBMP having reduced levels of non-osteogenic proteins or peptides may be combined with BMP, BPP, bisphosphonates, hormone therapy treatments, such as estrogen receptor modulators, calcitonin, vitamin D, calcium supplementation, PTH (such as Forteo or teriparatide, Eli Lilly), sodium fluoride and/or growth factors that have a positive effect on bone, such as

insulin-like growth factors I and II and transforming growth factor beta. Those skilled in the art would be able to determine the accepted dosages for each of the therapies using standard therapeutic dosage parameters, and reduced dosages of DBM and/or BMP where the effects of secondary agents are synergistic.

[0043] FIG. 4 depicts a flowchart of one method according to this invention. In this embodiment of the method, mammalian cells, such as mesenchymal stem cells can be harvested from a patient or a cell donor (100). The cells may be injected where bone formation or repair is desired, or first treated with at least one agent (such as extracted DBM, BMP or proteins) to induce osteogenesis or specifically osteoblastic differentiation (102). The cells may then be administered to a patient, such as systemically or at a selected site at which osteogenesis is desired (104). Additionally, the patient may be treated locally or systemically with at least one additional agent which effects osteogenesis, such as DBM and nBMP alone or in combination with BMP-2, BMP-4, BMP-7 or other osteogenic factors (106), or BPP to enhance the osteogenic activity of BMP-2, for example.

[0044] FIGS. 5A & B depict two embodiments of the present invention. In FIG. 5A, the invention may include implants or grafts (200) for use *in vivo* comprising, a substrate having a surface (201), wherein at least a portion of the surface of the implant includes extracted DBM, nBMP and/or an extract of non-collagenous proteins recovered from DBM extraction (203) in an amount sufficient to induce osteogenesis, chondrogenesis, or calcification in the surrounding tissue, and the implant may include osteogenic cells expressing BBP and/or BMP. The implant may be shaped in the form of pins, screws, plates or prosthetic joints which may be placed in the proximity of, or in contact with a bone (202) that are used to immobilize a fracture, enhance bone formation, or stabilize a prosthetic implant by stimulating formation or repair of a site of bone removal, fracture or other bone injury (204).

[0045] As shown in FIG. 5B, the invention may also include the *in vivo* or *in vitro* (such as on cultures of collagen or chondrocytes) or *in vivo* application of at least an extracted DBM and/or nBMP containing composition (206) in the proximity of or in contact with a bone (202), an implant (200) at a site of bone removal, fracture or other bone injury (204) where osteogenesis or calcification is desired.

The extracted DBM, nBMP and/or an extract of non-collagenous proteins recovered from DBM extraction may be applied in combination with other agents such as BBP, BMP-2, BMP-4, BMP-7 or collagen cultures. For example: stem cells for treating bone related disorders in humans has also been examined. Infusion of osteoblastic progenitor stem cells from a healthy individual into a diseased individual has been shown to improve bone density in some osteogenic insufficient patients.

[0046] FIG. 6 depicts a flowchart of one method according to this invention. For example, DBM may be obtained from bone using methods known to those in the art. The DBM may be extracted in a alkali-urea solution, and the alkali-urea-DBM solution may be adjusted, such as to pH about 10.5. The DBM may be separated from the alkali-urea solution, and optionally, nBMP may be produced from resulting the DBM using methods known to those of skill in the art. The DBM or nBMP may be further extracted to derive non-collagenous proteins with osteogenic activity. The resulting DBM or nBMP may be used, such as for implanting *in vivo* at a site where bone formation is desired.

EXAMPLE 1:

[0047] A series of studies were undertaken in which chemical extractions were tested for the potential to remove enhancers or inhibitors of BMP activity from DBM. One such treatment was an overnight extraction with alkali-urea, which was used to remove proteins from DBM based on the net charge of the proteins. The osteogenic activity of extracted DBM was compared to that of DBM to which an equal mass of concentrated extractate had been added.

MATERIALS AND METHODS

Production Of Demineralized Bone Matrix. Demineralized bone matrix (DBM) was prepared as described previously by Urist (Urist, M.R., Huo, Y.K., Brownell, A.G., Hohl, W.M., Buyske, J., Lietze, A., Tempst, P., Hunkapiller, M., and DeLange, R.J. (1984) Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography. Proc. Natl. Acad. Sci. U.S.A. 81:371-375. Urist, M.R. Emerging Concepts of Bone Morphogenetic Protein. (1991) In: Fundamentals of Bone Growth: Methodology and Applications, A.D. Dixon, B.G. Sarnat, and D.A.N. Hoyte (eds.), pp. 189-198. (C.R.C. Press, Boston). Briefly, fresh bone was cleaned, ground, washed with water in the presence of protease

inhibitors, defatted with 1:1 (v/v) chloroform: methanol, demineralized with 0.6 M HCl, washed again, and lyophilized.

[0048] *Alkali-Urea Extraction Of Demineralized Bone Matrix.* DBM was extracted in a solution which had been prepared by adding equal volumes of 6 M urea and 0.1 M KOH. The pH of this solution was adjusted to 10.5 with HCl and the solution was made 2 mM N-ethylmaleimide (NEM), 0.1 mM benzamidine HCl, and 0.02% NaN₃. Ground DBM was left in this solution in the cold overnight with stirring. A proportion of 5 liters/kg was used. The pH of the solution was adjusted to 10.5 with 1M KOH at 0.5, 1, and 4 hours, but not subsequently. The following day the supernatant was removed by decanting and the remaining solid material was then washed with a large volume of water containing protease inhibitors in the cold overnight, and subsequently collected by filtration and lyophilized. The extracted supernatant was collected and dialyzed against 20 volumes of water in the cold with 2 changes over a 48 hour period. Dialysis tubing with a 6-8 kD molecular weight cut-off was employed (Spectropor from Spectrum Medical Industries, Laguna Hills, CA). The dialyzed supernatant was centrifuged at 10,000 rpm for 30 minutes at 4° C in a Sorvall GSA rotor (Kendro Laboratory Products, Newtown, CT). The precipitate and supernatant were lyophilized separately. The water soluble fraction was used in subsequent experiments.

[0049] *In Vivo Assay Of Osteogenic Activity.* The osteogenic activity of test materials were tested using an *in vivo* assay approved by the Sepulveda Animal Subjects Committee and the VA Greater Los Angeles Research and Development Committee. Male NIH Swiss-Webster nude mice aged 5 to 8 weeks were used (Taconic Farms, Germantown, NY). Prior to the assay, the DBM to be tested was sorted through number 100 and number 20 wire sieves. Material with a particles size of 150 to 850 micrometers was placed in #5 gelatin capsules and sterilized by exposure to chloroform vapor. To conduct the assay, mice were anesthetized using 1% isoflurane delivered in oxygen at 2 l/min through a small animal anesthesia machine (VetEquip, Pleasanton, CA). Animals were affixed to a surgery board and the fur over the hindquarters was shaved. The skin was cleaned with 70% ethanol and a midline incision made over the spine adjacent to the hindquarters. Blunt dissection with scissors was used to expose the quadriceps muscle on one side. A small pouch was made in

the muscle using the point of the scissors and the #5 capsule containing the test material was inserted into the pouch. The skin was then closed with three 11-mm Michel surgical clips and the animal returned to its cage for monitoring. After 28 days the animals were sacrificed and the hindquarter removed. Radiological examination of the specimens was accomplished using a small parts X-Ray cabinet (Faxitron, Wheeling, IL). Specimens were then placed in buffered formalin and submitted for routine processing for histological examination. Implants consisted of 25 mg of starting material DBM, 25 mg of extracted DBM, or 25 mg of extracted DBM plus 25 mg of concentrated water soluble extractate.

[0050] *Electrophoresis And Western Blotting.* SDS polyacrylamide gel electrophoresis under non-reducing conditions and Western blotting were conducted employing standard equipment and the instructions provided by the manufacturer (BioRad, Hercules, CA). Noggin/Fc recombinant chimeric protein and goat anti-mouse noggin were obtained from R & D Systems (Minneapolis, MN). The primary antibody was used at final concentration of 0.075 µg/ml. The secondary antibody was donkey anti-goat IgG conjugated to alkaline phosphatase and was used at a 1:2500 dilution (Pierce, Rockford, IL). Color development was accomplished with the BCIP/NBT with suppressor reagent from Pierce (Rockford, IL).

[0051] In order to determine the relative noggin content of the DBM starting material, extracted DBM, and the extractate, 400 µl of Laemmli sample buffer was added to 50 mg of each material. The samples were boiled, mixed vigorously, and briefly spun in a centrifuge. Aliquots of 20 µl, representing about 2.5 mg of material, were separated by SDS-PAGE electrophoresis. Parallel gels were run and stained with Coomassie blue in order to confirm equal loading.

RESULTS

[0052] *The Effect of Extractate on the Osteogenic Activity of DBM.* Figures 1 and 2 demonstrate radiologically the effect of an equal amount (25 mg) of water soluble extractate on the osteogenic activity of DBM. The results presented in Figure 1 confirm and compare the osteogenic activity of DBM starting material (Figure 1, two specimens on the left) and extracted DBM (Figure 1, two specimens on the right). Both DMB and extracted DMB induced ectopic bone formation as can be seen in the large areas of calcification (shown at arrows).

Histological examination confirmed that the calcification was associated with osteogenic bone formation and not simply dystrophic calcification.

[0053] The results presented in Figure 2 show the effects of the water soluble extractate on the osteogenic potency of extracted DBM. No ectopic bone formation was observed in any of the four implants containing both extracted DBM (25 mg) and the water soluble fraction of the alkali-urea extractate (25 mg) (Figure 2, all four specimens).

[0054] *Demonstration of the Presence of Noggin, an Inhibitor of BMPs, in the Water Soluble Extractate.* Figure 3 shows a Western blot of DBM, extracted DBM, extractate, and recombinant noggin/Fc chimeric protein developed with an anti-mouse noggin primary antibody. The M_r of the noggin/Fc chimeric protein is 60 kD whereas the expected M_r of native (dimeric) noggin is 64 kD (Smith, W.C., et al. (1993). Secreted noggin protein mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm. *Nature* 361:547-549). This study confirms the presence of noggin, a known inhibitor of BMP activity, generally, in the water soluble fraction of the alkali-urea extractate of DBM (Figure 3, lane A) at a much higher level than in the DBM starting material (Figure 3, lane C) and especially the extracted DBM (Figure 3, lane B).

[0055] While the specification describes particular embodiments of the present invention, those of ordinary skill can devise variations of the present invention without departing from the inventive concept.

[0056] Claims:

- [0057]** 1. A composition comprising a demineralized bone matrix having been extracted with a chaotropic solvent, wherein the extracted demineralized bone matrix has osteoinductive activity, and wherein the extracted demineralized bone matrix has less non-osteogenic proteins than native demineralized bone matrix.
- [0058]** 2. The composition of claim 1, wherein the chaotropic solvent comprises urea in a concentration between about 1M to about 4.5M.
- [0059]** 3. The composition of claim 1, wherein the chaotropic solvent comprises urea in a concentration between about 6M urea and 0.1M KOH.
- [0060]** 4. A composition comprising noncollagenous proteins having been extracted from a first extraction of demineralized bone matrix with a chaotropic solvent, and a second extraction with chaotropic solvents, ionic detergents or non-denaturing concentrations of acid, wherein the noncollagenous protein has osteogenic activity and less non-osteogenic proteins than an equal amount of non-extracted protein.
- [0061]** 5. The composition of claim 3 wherein the second extraction chaotropic solvent comprises about 4M guanidine HCL and about 6M urea.
- [0062]** 6. The composition of claim 3 wherein the second extraction ionic detergent comprises about 1% sodium dodecylsulfate.
- [0063]** 7. The composition of claim 3 wherein the second extraction acid comprises about 1-2M citric acid.
- [0064]** 8. A composition having osteogenic activity comprising demineralized bone matrix or native BMP, substantially free of non-osteogenic proteins.
- [0065]** 9. A composition having osteogenic activity comprising demineralized bone matrix or native BMP, having less osteogenic inhibiting proteins than native DBM or BMP.
- [0066]** 10. A composition having osteogenic activity comprising demineralized bone matrix or native BMP, having less noggin than an equal amount of native DBM or BMP.
- [0067]** 11. A composition comprising demineralized bone matrix or BMP, having a reduced level of or substantially free of inhibitory proteins, such as BMP noggin.

[0068] 12. The composition of claims 1, 2 or 3, further including at least one of BMP-2, BMP-4 or BMP-7.

[0069] 13. A method for producing DBM having reduced levels of osteogenic inhibitory factors comprising the steps of:

- (a) obtaining DBM from bone tissue;
- (b) extracting the DBM with chaotropic solvent;
- (c) isolating the resulting DBM.

[0070] 14. A medicament for use in enhancing bone formation comprising a therapeutically effective dosage of DBM or BMP or having reduced levels of BMP inhibitors, relative to an equal amount native DBM or BMP.

[0071] 15. The medicament of claim 14, further comprising at least one agent selected from the group comprising parathyroid hormone, sodium fluoride, insulin-like growth factor I, insulin-like growth factor II or transforming growth factor beta, bisphosphonates, estrogen receptor modulators, calcitonin, vitamin D or calcium.

[0072] 16. The medicament of claim 14, further comprising a therapeutically effective dosage of at least one of BMP-2, BMP-4 or BMP-7.

[0073] 17. A method of inducing osteogenesis including treating mammalian cells with DBM or nBMP substantially free of noggin.

[0074] 18. The method of claim 17, further comprising treating mammalian cells with at least one of BMP-2, BMP-4 or BMP-7.

[0075] 19. A method of inducing osteogenesis including treating a mammalian cell with DBM or nBMP having reduced levels of noggin relative to an equal amount of native DBM or nBMP.

[0076] 20. The method of claim 19, further comprising treating the mammalian cell with at least one of BMP-2, BMP-4 or BMP-7.

[0077] 21. The method of claim 19, further comprising treating the mammalian cells with at least one agent selected from the group comprising parathyroid hormone, sodium fluoride, insulin-like growth factor I, insulin-like growth factor II or transforming growth factor beta, bisphosphonates, selective estrogen receptor modulators, calcitonin, vitamin D, or calcium, at a therapeutically effective dose.

[0078] 22. A method of stimulating mammalian cells to express a level of a biological marker of osteogenesis which is greater than the level of a biological marker in untreated cells, comprising exposing a mammalian cells to DBM or nBMP having been extracted with an chaotropic solvent and having reduced levels of osteogenic inhibiting protein relative to an equal amount non-extracted noggin relative to native DBM or BMP.

[0079] 23. The method of claim 22, further comprising treating the mammalian cell with at least one of BMP-2, BMP-4 or BMP-7.

[0080] 24. The method of claim 22 wherein the biological marker is an increase in at least one of alkaline phosphatase activity, calcium incorporation, mineralization or expression of osteocalcin mRNA.

[0081] 25. A method of inducing bone formation comprising: administering osteogenic cells to a location proximate to the desired location of osteogenesis; and administering DBM or BMP having reduced levels of noggin relative to native DBM or nBMP, at a location proximate to the desired location of osteogenesis.

[0082] 26. The method of claim 25, further comprising administering at least one of BMP-2, BMP-4 or BMP-7.

[0083] 27. An article of manufacture comprising a support, wherein said support comprises DBM or BMP, or wherein said DBM or nBMP has reduced levels of noggin relative to native DBM or BMP, and said DBM or BMP has osteogenic activity.

[0084] 28. An article of manufacture of claim 27, further comprising a protein immobilized on a solid support, wherein said protein comprises at least one of BMP-2, BMP-4, BMP-7 or fragments thereof, wherein said fragment has osteogenic activity.

[0085] 29. An implant comprising, a substrate having a surface, wherein at least a portion of the surface of the implant comprises DBM or BMP having reduced levels of noggin relative to equal amounts of native DBM or nBMP, in an amount sufficient to enhance osteogenesis.

[0086] 30. The implant of claim 29, further comprising at least one of BMP-2, BMP-4 or BMP-7.

[0087] 31. The implant of claim 29, wherein the substrate is formed into the shape of a pin, screw, plate, or prosthetic joint.

[0088] 32. The implant of claim 29, wherein at least the surface of the implant further includes osteogenic mammalian cells.

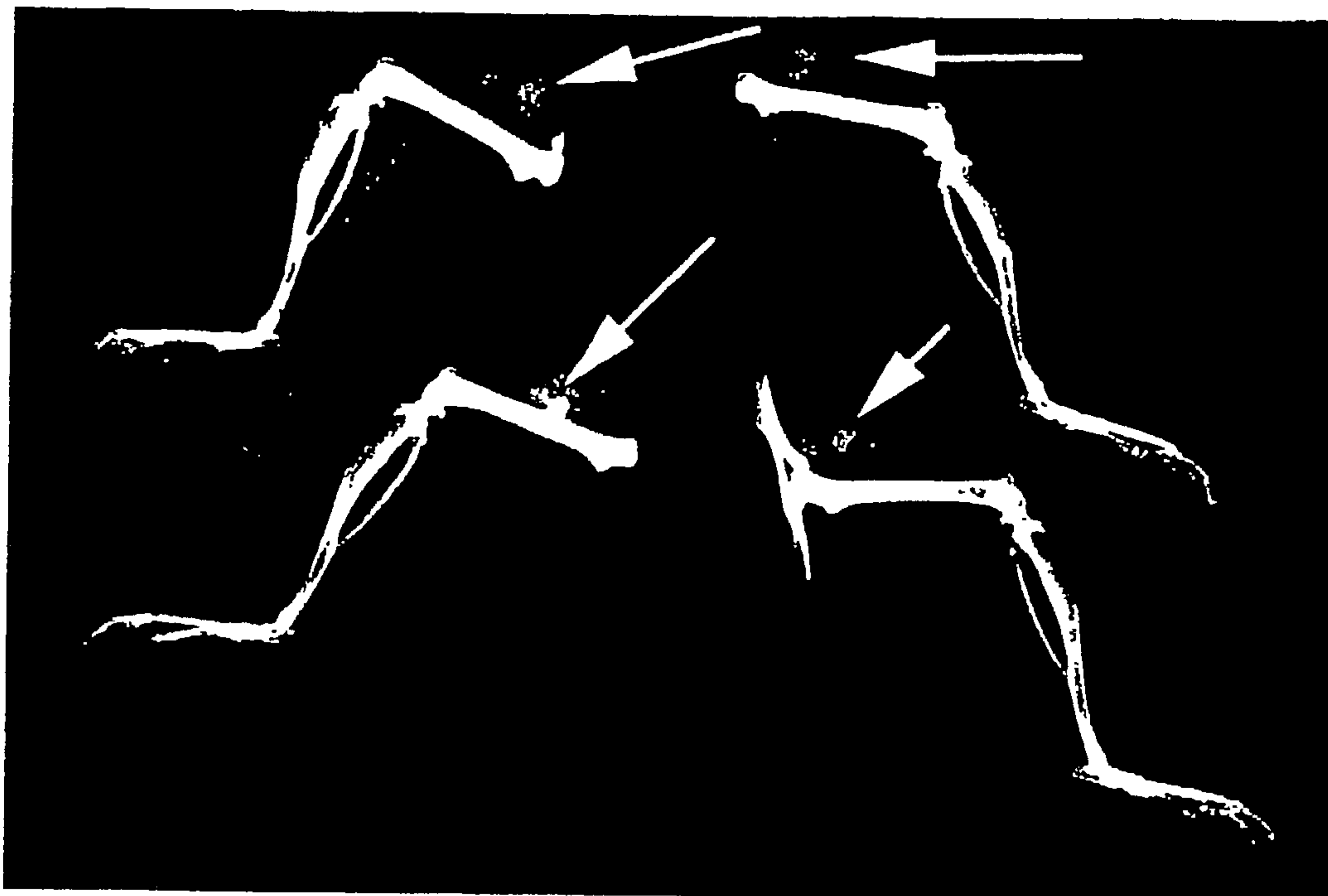
[0089] 33. A method of producing a composition having increased levels of osteogenic inhibiting factors:

- (a) obtaining DBM from bone tissue;
- (b) extracting the DBM with chaotropic solvent;
- (c) isolating the water soluble fraction including osteogenic inhibiting factors.

[0090] 34. The method of claim 33 wherein the osteogenic inhibiting factor is noggin.

[0091] 35. A composition comprising proteins for inhibiting the osteogenic activity of DBM, having been obtained from the water soluble extractate from an extraction of demineralized bone matrix with a chaotropic solvent.

FIG. 1 $\frac{1}{4}$



DMB

Extracted DMB

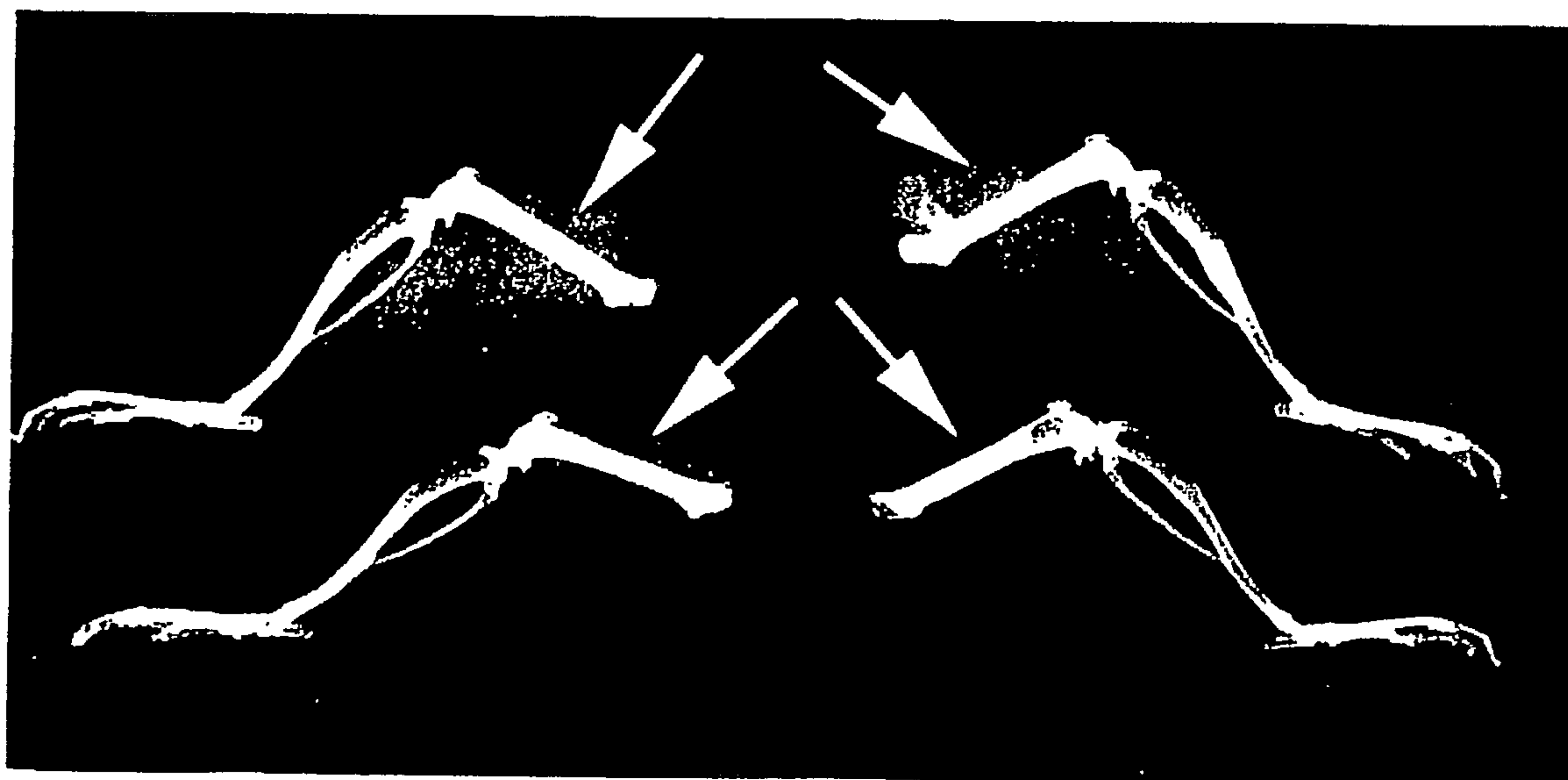


FIG. 2

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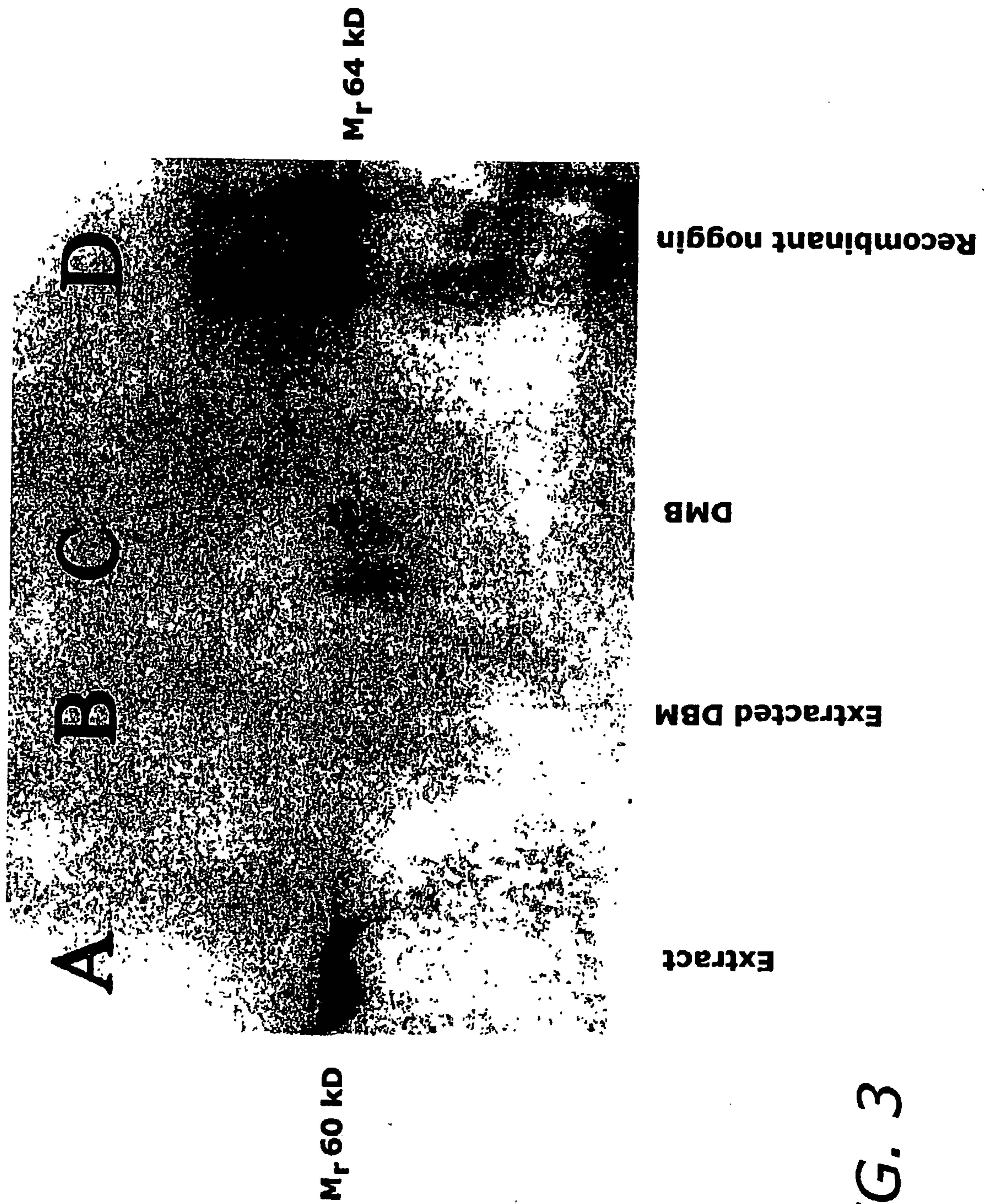
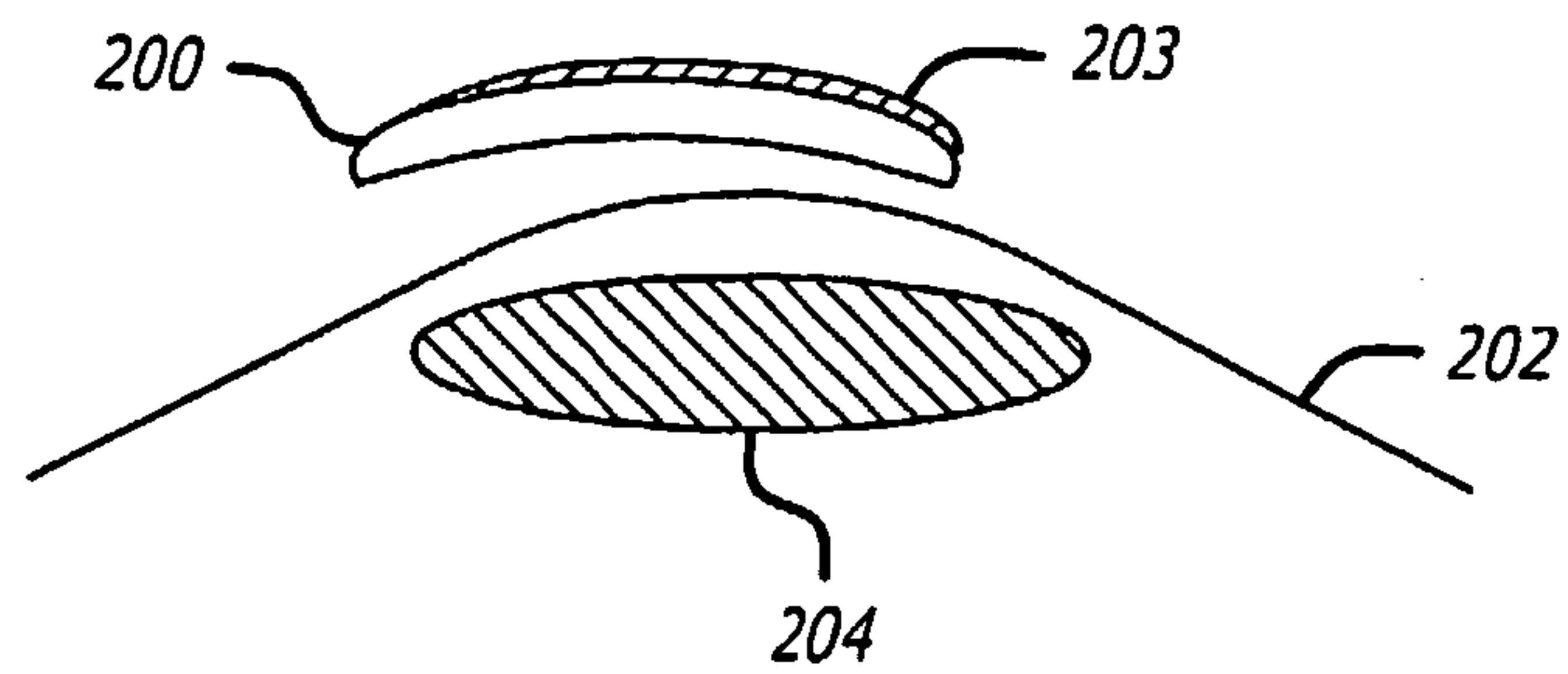
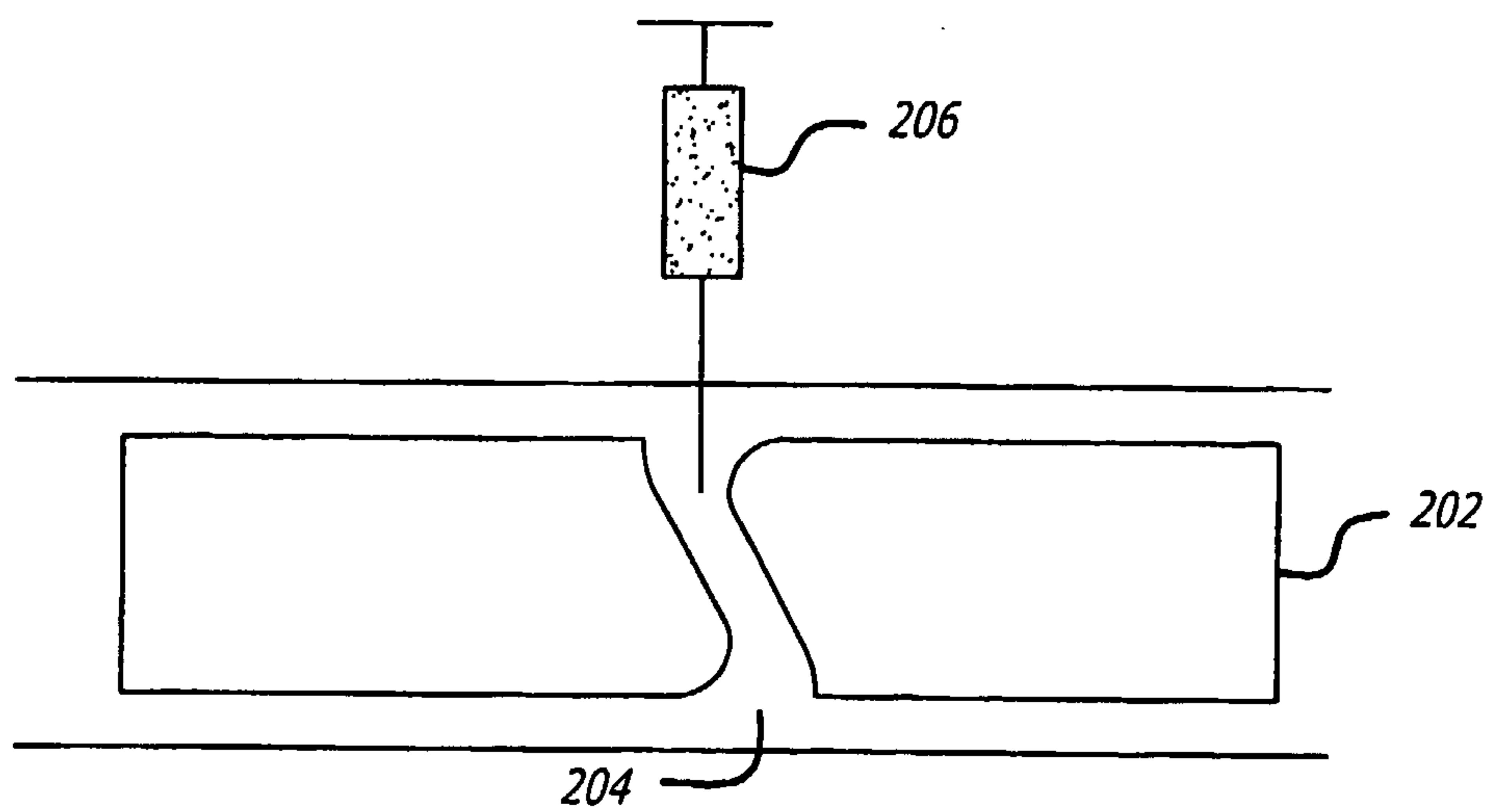


FIG. 3

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*FIG. 5A**FIG. 5B*

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FIG. 4

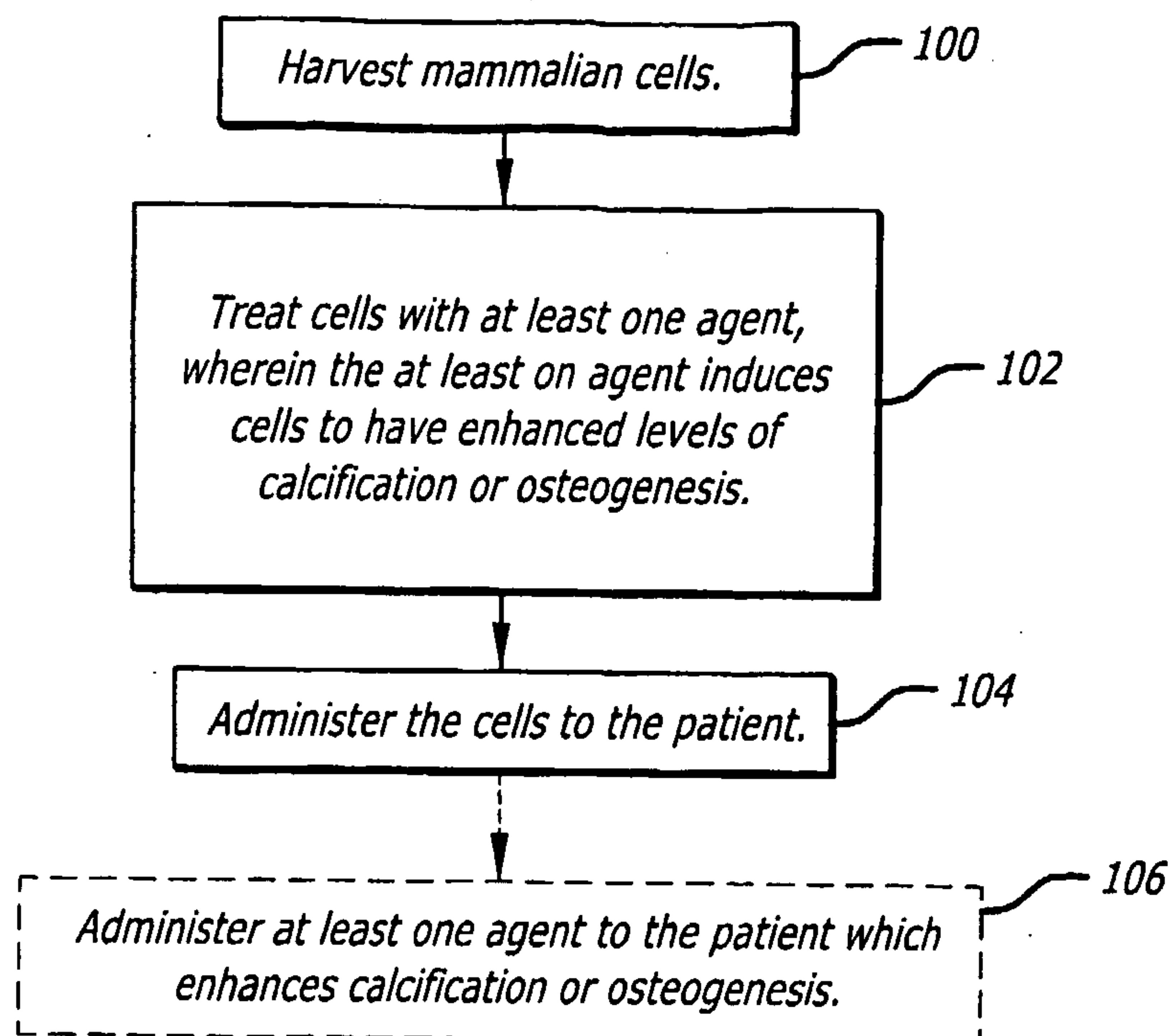
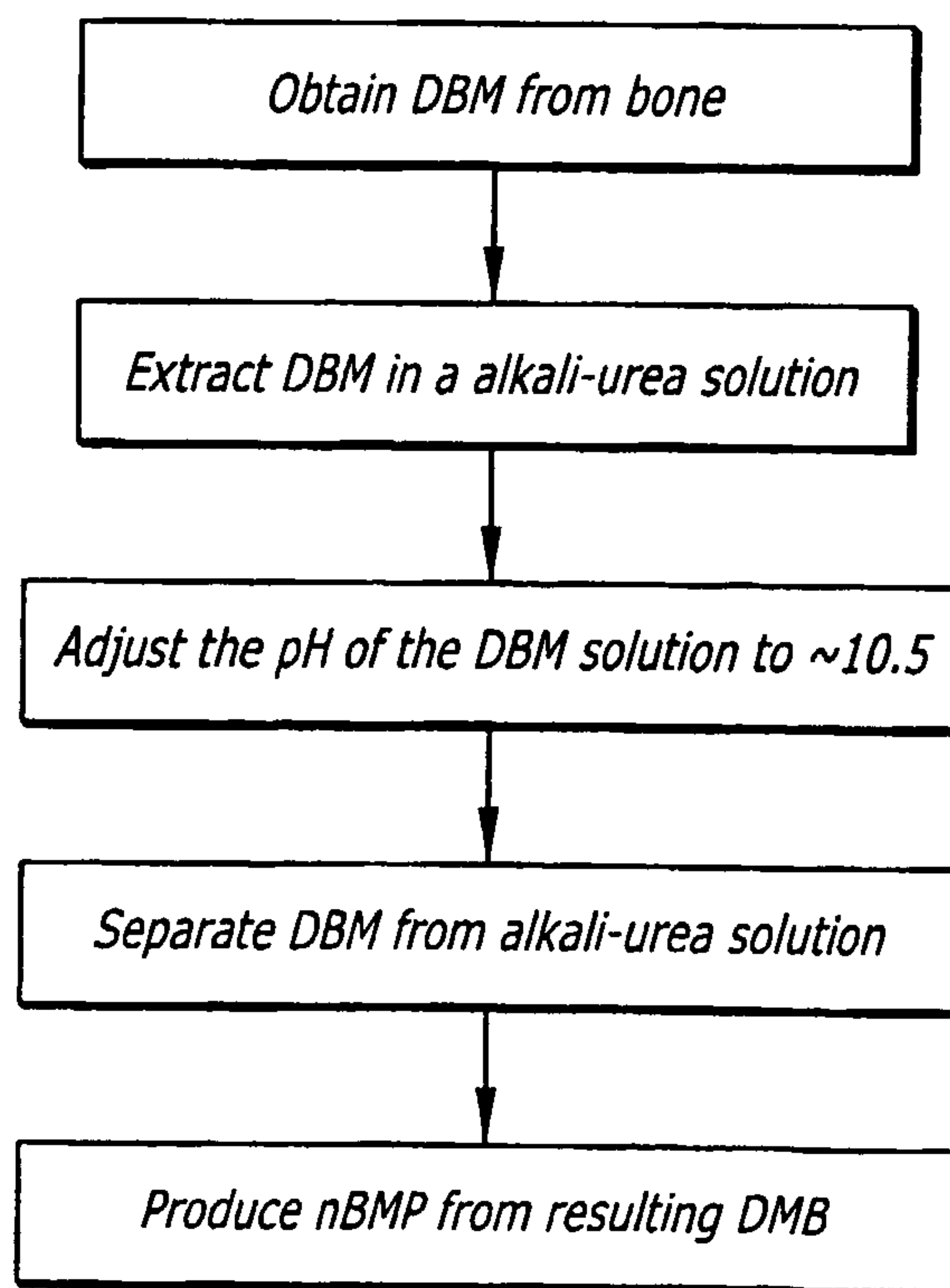
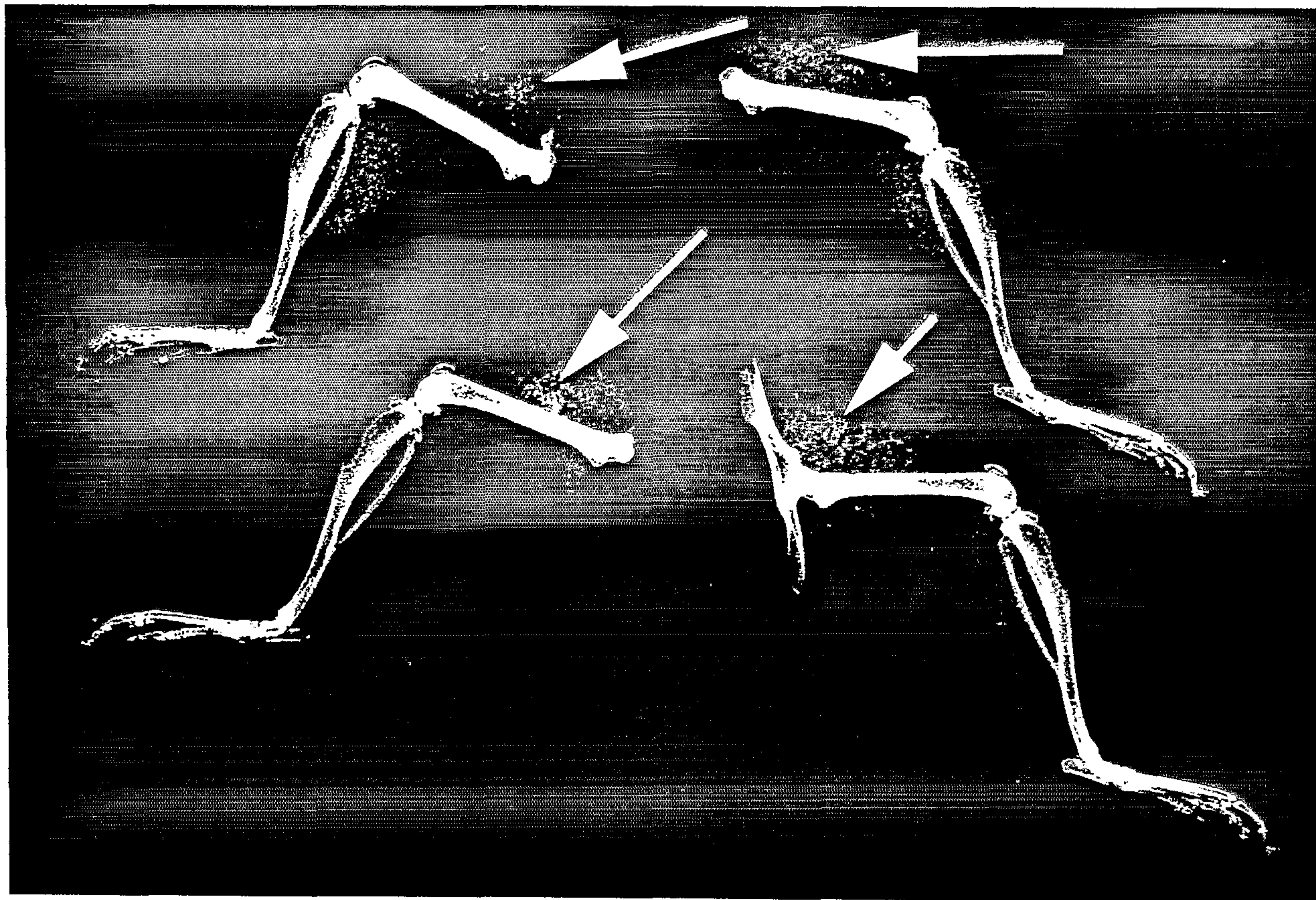


FIG. 6





DMB

Extracted DMB