Notice

DESCRIPTION CN105435307A

Decellularized and decalcified bone materials derived from natural tissues and their preparation methods

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天然组织来源的脱细胞联合脱钙骨材料及其制备方法

[0001]

Technical Field

技术领域

[0002]

This invention belongs to the field of bone tissue repair and regeneration technology, and particularly relates to a decellularized and decalcified bone material derived from natural tissue and its preparation method.

本发明属于骨组织修复及其再生技术领域，尤其涉及一种天然组织来源的脱细胞 联合脱钙骨材料及其制备方法。

[0003]

Background Technology

背景技术

[0004]

Currently, osteonecrosis and bone defects caused by factors such as trauma, tumors, and infections are very common in clinical practice. As a result, artificial biomaterials have been gradually used for the repair of patients' bone tissue.

目前，外伤、肿瘤、感染等因素导致的骨坏死、骨缺损在临床上十分常见，为此，人 工生物材料已逐步运用于病人的骨组织修复。

However, due to its shortcomings in biocompatibility, degradability, osteoconductivity, and osteoinduction, autologous bone grafting is still considered the most ideal bone repair material.

但是，由于生物相容性、降解性、骨传导性、骨 诱导方面等不足，使自体骨移植仍然被视为最理想的骨修复材料。

In actual clinical surgery, due to issues such as the amount of material to be harvested and secondary damage, autologous bone transplantation cannot adequately meet the needs of repairing and treating osteonecrosis or bone defects in patients.

而在实际临床手术中，由 于取材数量和二次损伤等问题，自体骨移植并不能很好满足于病人骨坏死或骨缺损的修复 和治疗。

[0005]

Natural tissue-derived extracellular matrix (ECM) contains various biochemical factors required by normal tissue or organ cells and has a natural macroscopic and ultramicro three-dimensional structure.

天然组织来源的细胞外基质(ECM)含有正常组织或器官细胞所需的各种生化因 子，并且具有天然宏观及超微三维的立体结构。

Currently, the use of decellularized cancellous and compact bone materials derived from animals (cattle, pigs) and allogeneic humans for fracture repair and bone defect filling in clinical patients is in the preliminary exploratory stage.

目前，运用动物(牛、猪)和异体人来源的脱 细胞松质骨和密质骨材对临床上病人的骨折修复和骨缺损填补正处于初步探索阶段。

However, due to factors such as the sampling site, type of bone tissue, and size of bone tissue, bone decellularization protocols vary greatly among different companies or laboratories. Different intensities of decellularization methods can result in significant differences in the retention of the extracellular matrix, leading to a lack of uniformity in bone repair outcomes among patients in clinical practice. Meanwhile, existing technologies often use strong acid decalcification (concentrated hydrochloric acid, formic acid, acetic acid, etc.) to obtain decalcified bone tissue or decellularized decalcified bone material. This process greatly reduces the biological activity of ECM, causing the bone material to largely lose its natural biological ability to repair bone.

然 而，由于取材部位、骨组织类别、骨组织大小等问题，使不同公司或实验室的骨脱细胞方案 有着很大的差别。 而不同强度的脱细胞方法则会对细胞外基质的保留产生很大的差异，这 就使临床上病人骨修复效果缺乏均一性。 同时，现有技术对骨组织往往采取强酸脱钙(浓盐 酸、甲酸、乙酸等)的方法获得脱钙骨组织或脱细胞脱钙骨材料，该处理工艺会大大降低ECM 的生物学活性，使骨材料很大程度上丧失了修复骨的天然生物能力。

[0006]

Summary of the Invention

发明内容

[0007]

The technical problem to be solved by the present invention is to provide a decellularized and decalcified bone material derived from natural tissue and its preparation method. This method can simultaneously and completely decellularize both cancellous bone and compact bone, and the conditions are mild, without ECM damage, and are rapid and stable.

本发明要解决的技术问题是提供一种天然组织来源的脱细胞联合脱钙骨材料及 其制备方法，该法能同时对松质骨和密质骨进行完全去细胞化处理，且条件温和、无ECM损 害、快速稳定。

[0008]

To solve the above problems, the present invention adopts the following technical solution: a method for preparing decellularized and decalcified bone material from natural tissue, wherein arbitrary mammalian bone tissue is treated with physiological saline buffer containing protease inhibitors, organic solvent solution, PBS buffer containing Triton X, PBS buffer containing SDS, PBS buffer containing trypsin, PBS buffer containing DNase, EDTA isotonic solution, and ultrasound to obtain decellularized and decalcified bone material.

为解决上述问题，本发明采用以下技术方案：天然组织来源的脱细胞联合脱钙骨 材料的制备方法，将哺乳动物任意骨组织经含蛋白酶抑制剂的生理盐水缓冲液、有机溶剂 溶液、含TritonX的PBS缓冲液、含SDS的PBS缓冲液、含胰酶的PBS缓冲液、含DNA酶的PBS缓 冲液、EDTA等渗液和超声波处理，获得脱细胞、脱钙的骨材料。

[0009]

Any bone tissue in mammals includes the long bones of the limbs, vertebrae, and iliac bones.

哺乳动物任意骨组织为四肢长骨、椎骨、髂骨。

[0010]

Physiological saline buffer containing protease inhibitors, with a sodium chloride concentration of 1%-5% and a protease inhibitor concentration of 10 KIU/ml:

含蛋白酶抑制剂的生理盐水缓冲液，氯化钠浓度为1%-5%，蛋白酶抑制剂含量为 10KIU/ml：

[0011]

The organic solvent solution is a chloroform and methanol solution in equal volume ratio, an acetone solution with a volume concentration of 10%-100%, or an ethanol solution with a volume concentration of 30%-70%.

有机溶剂溶液为等体积比的氯仿和甲醇溶液、体积浓度10%-100%的丙酮溶液或 体积浓度30%-70%的乙醇溶液；

[0012]

The PBS buffer containing Triton X is a PBS buffer containing 1%-10% Triton X-200 or Triton X-100 by volume.

含TritonX的PBS缓冲液为体积浓度1%-10%的TritonX-200或TritonX-100的 PBS缓冲液；

[0013]

The PBS buffer containing SDS is a PBS buffer with a mass concentration of 0.5%-10% SDS, mixed with 1-20 mmol/L Tris;

含SDS的PBS缓冲液为质量浓度0.5%-10%的SDS的PBS缓冲液，其中混合1- 20mmol/L的Tris；

[0014]

The concentration of trypsin in PBS buffer containing trypsin is 0.25%-5%;

含胰酶的PBS缓冲液中胰酶浓度为0.25%-5%；

[0015]

The concentration of DNase in PBS buffer containing DNase is 0.01-0.5 mg/ml;

含DNA酶的PBS缓冲液中DNA酶浓度为0.01-0.5mg/ml；

[0016]

The concentration of EDTA isotonic solution is 0.1%-10%.

EDTA等渗液浓度为0.1%-10%。

[0017]

The preparation method of the above-mentioned decellularized and decalcified bone material from natural tissue sources includes the following steps:

上述天然组织来源的脱细胞联合脱钙骨材料的制备方法，包括以下步骤：

[0018]

(1) Take any bone tissue from the whole body of mammals and rinse it three times with sterile physiological saline for 20 minutes each time to remove blood, residual muscle tissue, hair and ligaments, etc.

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(1)取哺乳动物全身任意骨组织用无菌生理盐水漂洗3次、20分钟/次，去除血液、 残余肌肉组织、毛发和韧带等组织；

[0019]

(2) In physiological saline buffer containing protease inhibitor, shake at 150 rpm for 8-24 hours at a constant temperature of 45°C;

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(2)在含蛋白酶抑制剂的生理盐水缓冲液中，恒温45°C摇床150rpm震荡8-24小时；

[0020]

(3) Degrease in an organic solvent solution at a constant temperature of 45°C and a shaking speed of 150rpm for 2-12 hours;

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(3)在有机溶剂溶液中，恒温45°C摇床150rpm震荡脱脂2-12小时；

[0021]

(4) Add penicillin and streptomycin mixed antibacterial solution to PBS buffer containing Triton X, and shake at 150 rpm for 3-72 hours at a constant temperature of 45°C.

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(4)在含TritonX的PBS缓冲液中，加入青霉素和链霉素混合抗菌液，恒温45°C摇 床150rpm震荡3-72小时；

[0022]

(5) Add penicillin and streptomycin mixed antibacterial solution to PBS buffer containing SDS, and shake at 150 rpm for 2-96 hours at a constant temperature of 45°C.

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(5)在含SDS的PBS缓冲液中，加入青霉素和链霉素混合抗菌液，恒温45°C摇床 150rpm震荡2-96小时；

[0023]

(6) Add penicillin and streptomycin mixed antibacterial solution to PBS buffer containing trypsin, and shake at 150 rpm for 1-12 hours at 37°C;

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(6)在含胰酶的PBS缓冲液中，加入青霉素和链霉素混合抗菌液，37°C摇床150rpm 震荡1-12小时；

[0024]

(7) Add penicillin and streptomycin mixed antibacterial solution to PBS buffer containing DNase, and shake at 37°C and 150 rpm for 1-12 hours;

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(7)在含DNA酶的PBS缓冲液中，加入青霉素和链霉素混合抗菌液，37°C摇床150rpm 震荡1-12小时；

[0025]

(8) Treat the solution in EDTA isotonic solution with an ultrasonic machine with a power of 20-200W for 2-24 hours.

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(8)在EDTA等渗液中，以功率20-200W的超声波机器处理2-24小时；

[0026]

(9) The decellularized and decalcified bone material derived from natural tissue was obtained by shaking at 37°C and 150 rpm for 72 hours in sterile saline.

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(9)在无菌生理盐水中，37°C摇床150rpm震荡72小时，即得天然组织来源的脱细胞 联合脱钙骨材料。

[0027]

The concentrations of penicillin and streptomycin in the mixed antibacterial solution are 10 KIU/ml and 10 KIU/ml, respectively, and the ratio of penicillin to streptomycin is 1:1; the volume ratios of PBS buffer and mixed antibacterial solution in steps (4)-(7) are 10:1, 10:1, 5:1, and 5:1, respectively.

混合抗菌液中青霉素和链霉素的浓度分别为10KIU/ml、10KIU/ml，青霉素和链霉 素的比例为1：1；步骤(4)-(7)中PBS缓冲液和混合抗菌液体积比分别为10：1、10：1、5：1、5： 1。

[0028]

In steps (2)-(7), rinse with saline solution for 5 hours after each step is completed.

步骤(2)-(7)中，每个步骤完成后均用生理盐水冲洗5小时。

[0029]

The above preparation method yields decellularized and decalcified bone material derived from natural tissues.

上述制备方法得到的天然组织来源的脱细胞联合脱钙骨材料。

[0030]

To address the problems existing in current bone materials and their preparation methods used for bone repair and regeneration, the inventors have established a method for preparing decellularized and decalcified bone materials from natural tissue sources. This method involves treating arbitrary mammalian bone tissue with physiological saline buffer containing protease inhibitors, organic solvent solutions, PBS buffer containing Triton X, PBS buffer containing SDS, PBS buffer containing trypsin, PBS buffer containing DNase, EDTA isotonic solution, and ultrasound to obtain decellularized and decalcified bone materials.

针对目前用于骨修复和再生的骨材料及其制备方法存在的问题，发明人建立了一 种天然组织来源的脱细胞联合脱钙骨材料的制备方法，该法将哺乳动物任意骨组织经含蛋 白酶抑制剂的生理盐水缓冲液、有机溶剂溶液、含TritonX的PBS缓冲液、含SDS的PBS缓冲 液、含胰酶的PBS缓冲液、含DNA酶的PBS缓冲液、EDTA等渗液和超声波处理，获得脱细胞、脱 钙的骨材料。

This invention can simultaneously and completely decellularize both cancellous and compact bone under mild conditions, without ECM damage, and is rapid and stable. The resulting decellularized and decalcified bone material has advantages such as good biocompatibility, strong plasticity, and high biomechanical strength. It can be used to repair bone defects and bone nonunion caused by various clinical etiologies, as well as other bone regeneration and repair obstacles.

本发明能同时对松质骨和密质骨进行完全去细胞化处理，且条件温和、无ECM 损害、快速稳定，所得脱细胞联合脱钙骨材料具有生物相容性好、可塑性强、生物力学强度 高等优点，可用于修复临床上各类病因造成的骨缺损和骨不愈合等骨再生、修复障碍。

[0031]

The significant advancement of this invention compared to existing technologies lies in:

相对于现有技术，本发明的显著进步在于：

[0032]

(1) The present invention uses a decellularization scheme to simultaneously achieve thorough decellularization of any cancellous bone and compact bone in the whole body, and obtains highly uniform decellularized bone material more efficiently and conveniently.

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(1)本发明运用一种脱细胞方案即可同时实现对全身任意松质骨和密质骨进行彻 底去细胞化处理，更加高效便捷的获得均一性高的脱细胞骨材料。

[0033]

(2) This invention utilizes rapid and gentle EDTA combined with ultrasonic decalcification technology, which can greatly improve the biological activity and preparation speed of decellularized bone materials compared with traditional strong acid decalcification.

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(2)本发明运用快速而温和的EDTA联合超声波脱钙技术，较传统的强酸脱钙可大 大提高脱细胞骨材料的生物学活性以及制备速度。

[0034]

(3) While removing immunogenic allogeneic or xenogeneic cells, the present invention can preserve the integrity of the original ECM, and has good extracellular microenvironment,

biochemical factors and biomechanical properties, which can simulate the composition and structure of normal bone tissue to the greatest extent.

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(3)本发明在去除具有免疫原性的异体或异种细胞的同时，可保留原先ECM的完整 性，具有良好的细胞外微环境、生化因子和生物力学性质等，可以最大限度的模拟正常骨组 织的成分和结构。

[0035]

(5) This invention can customize individualized bone materials for patients, and can efficiently prepare decalcified materials to meet the complex and diverse bone repair and filling needs in clinical practice. It can also grind the materials into powder, dissolve the biochemical factors contained in normal bone tissue, and use them for orthopedic diseases in any part of the body.

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(5)本发明可以为病人订制具有个体化、可供移植的骨材料，并且高效制备脱钙材 料可满足临床上复杂多样的骨修复填补需求，也可以将其研磨制成粉末，将正常自身骨组 织中所含有的生化因子溶解，用于全身任意部位的骨科疾病。

[0036]

Attached Figure Description

附图说明

[0037]

Figure 1 shows HE staining of cancellous bone material, revealing cells and residual nuclear components (100x magnification).

图1是松质骨材料的HE染色无细胞及无细胞核成分残留图(放大100倍)。

[0038]

Figure 2 shows HE staining of dense bone material, revealing cells and residual nuclear components (100x magnification).

图2是密质骨质骨材料的HE染色无细胞及无细胞核成分残留图(放大100倍)。

[0039]

Figure 3 shows a DAPI fluorescence staining image of cancellous bone material with no residual nuclear components (magnified 100x).

图3是松质骨材料的DAPI荧光染色无细胞核成分残留图(放大100倍)。

[0040]

Figure 4 shows a DAPI fluorescence staining image of dense bone material with no residual nuclear components (magnified 100x).

图4是密质骨材料的DAPI荧光染色无细胞核成分残留图(放大100倍)。

[0041]

Figure 5 shows the DNA quantification detection of decellularized cancellous bone material.

图5是脱细胞松质骨材料的DNA定量检测图。

[0042]

Figure 6 shows the DNA quantification detection of decellularized compact bone material.

图6是脱细胞密质骨材料的DNA定量检测图。

[0043]

Figure 7 shows a 3D CT image of decellularized cancellous bone material used to repair a large defect in the upper and middle segment of a rabbit's tibia.

图7是脱细胞松质骨材料用于修复兔子胫骨中上段大面积缺损的三维CT图片。

[0044]

Figure 8 shows a 3D CT image of decellularized dense bone material used to repair a large defect in the upper and middle segment of a rabbit's tibia.

图8是脱细胞密质骨材料用于修复兔子胫骨中上段大面积缺损的三维CT图片。

[0045]

Detailed Implementation

具体实施方式

[0046]

Example 1: Preparation and Study of Completely Decalcified and Decellularized Cancellous Bone Material

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实施例1完全脱钙、脱细胞松质骨材料的制备和研究

[0047]

(1) Take cancellous bone from the pig iliac crest, transfer it to a 4°C environment, and rinse it three times with sterile saline for 20 minutes each time to remove blood, residual muscle tissue, hair, ligaments, and other tissues. The size of the bone sample depends on the actual needs of the clinical surgery, and is usually 2cm*2cm*2cm.

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(1)取猪髂骨松质骨，转移至4°C环境中，用无菌生理盐水漂洗3次、20分钟/次，去 除血液、残余肌肉组织、毛发和韧带等组织；取材大小视临床实际手术需要而定，通常大小 为2cm*2cm*2cm

[0048]

(2) In 1000 ml of physiological saline buffer containing protease inhibitor (concentration of 1%, protease inhibitor content of 10 KIU/ml), shake at 150 rpm for 24 hours at a constant temperature of 45°C, and rinse with physiological saline for 5 hours.

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(2)在1000ml含蛋白酶抑制剂的生理盐水缓冲液(浓度为1%，蛋白酶抑制剂含量 为10KIU/ml)中，恒温45°C摇床150rpm震荡24小时，并用生理盐水冲洗5小时；

[0049]

(3) In 1000 ml of chloroform and methanol solution of equal volume ratio, degrease at 45°C and shake at 150 rpm for 12 hours, and rinse with physiological saline for 5 hours.

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(3)在1000ml等体积比的氯仿和甲醇溶液中，恒温45°C摇床150rpm震荡脱脂12小 时，并用生理盐水冲洗5小时；

[0050]

(4) Add 100 ml of penicillin and streptomycin mixed antibacterial solution to 1000 ml of PBS buffer containing Triton X (1% Triton X-100), shake at 150 rpm for 72 hours at 45°C, and rinse with physiological saline for 5 hours; (5) Add 100 ml of penicillin and streptomycin mixed antibacterial solution to 1000 ml of PBS buffer containing SDS (0.5% SDS concentration, mixed with 1 mmol Tris), shake at 150 rpm for 96 hours at 45°C, and rinse with physiological saline for 5 hours;

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(4)在1000ml含TritonX的PBS缓冲液(浓度1%的TritonX-100)中，加入100ml青 霉素和链霉素混合抗菌液，恒温45°C摇床150rpm震荡72小时，并用生理盐水冲洗5小时；(5) 在1000ml含SDS的PBS缓冲液(SDS浓度为0.5%，混合1mmol的Tris)中，加入100ml青霉素和 链霉素混合抗菌液，恒温45°C摇床150rpm震荡96小时，并用生理盐水冲洗5小时；

[0051]

(6) Add 60 ml of penicillin and streptomycin mixed antibacterial solution to 300 ml of PBS buffer containing trypsin (trypsin concentration is 0.25%), shake at 150 rpm for 12 hours at 37° C, and rinse with physiological saline for 5 hours.

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(6)在300ml含胰酶的PBS缓冲液(胰酶浓度为0.25%)中，加入60ml青霉素和链霉 素混合抗菌液，37°C摇床150rpm震荡12小时，并用生理盐水冲洗5小时；

[0052]

(7) Add 60 ml of penicillin and streptomycin mixed antibacterial solution to 300 ml of PBS buffer containing DNase (DNase concentration is 0.01 mg/ml), shake at 150 rpm for 12 hours at 37°C, and rinse with physiological saline for 5 hours;

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(7)在300ml含DNA酶的PBS缓冲液中(DNA酶浓度为0.01mg/ml)，加入60ml青霉素和 链霉素混合抗菌液，37°C摇床150rpm震荡12小时，并用生理盐水冲洗5小时；

[0053]

(8) In 1000 ml of EDTA isotonic solution (concentration of 0.1%), treat with an ultrasonic machine with a power of 200 W for 24 hours;

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(8)在1000mlEDTA等渗液(浓度为0.1%)中，以功率200W的超声波机器处理24小 时；

[0054]

(9) The decellularized and decalcified bone material of natural tissue source was obtained by shaking in sterile saline at 37°C and 150 rpm for 72 hours. It was stored in liquid nitrogen and taken out during surgery.

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(9)在无菌生理盐水中，37°C摇床150rpm震荡72小时，即得天然组织来源的脱细胞 联合脱钙骨材料，液氮中保存，待手术时取出。

[0055]

The concentrations of penicillin and streptomycin in the mixed antibacterial solution were 10 KIU/ml and 10 KIU/ml, respectively, with a ratio of 1:1.

其中，混合抗菌液中青霉素和链霉素的浓度分别为10KIU/ml、10KIU/ml，青霉素和 链霉素的比例为1：1。

[0056]

Histological evaluation, quantitative detection of antigen components, and osteogenic repair capacity testing were performed on the completely decalcified and decellularized cancellous bone material obtained in this case. The results are shown in Figures 1, 3, 5, and 7.

对本例所得完全脱钙、脱细胞松质骨材料进行组织学评价、抗原成分定量检测和 成骨修复能力检测，结果如图1、3、5、7。

Figure 1, HE staining, shows that the extracellular matrix of the completely decalcified and decellularized cancellous bone material is intact, and the nuclear components are completely removed, with no cells or debris remaining. Figure 3, DAPI staining, further indicates that the nuclear components in the material are negative, and the antigenicity is completely removed. Figure 5, quantitative detection of DNA antigen components, shows that the DNA removal rate through decellularization can reach more than 95%. Figure 7, a rabbit tibia large-area bone defect repair experiment, shows that large-area bone defects were well repaired after using completely decalcified and decellularized cancellous bone material.

图1HE染色显示完全脱钙、脱细胞松质骨材料细胞 外基质保留完整，细胞核成分完全去除，无细胞及其碎片残留；图3DAPI染色进一步提示材 料中细胞核成分呈阴性，抗原性得到完全去除；图5的DNA抗原成分定量检测说明通过去细 胞DNA去除率可以达到95%以上；图7的兔子胫骨大面积骨缺损修复实验说明在使用了完全 脱钙、脱细胞松质骨材料后大面积大面积骨缺损得到了很好的修复。

[0057]

Example 2: Preparation and Study of Completely Decalcified and Decellularized Compact Bone Material

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实施例2完全脱钙、脱细胞密质骨材料的制备和研究

[0058]

Compact bone from the limbs of pigs is taken, and the size of the material is determined according to the actual needs of the clinical surgery, usually 1*1*0.5cm; the rest is carried out in accordance with the method of Example 1 to obtain completely decalcified and decellularized compact bone material.

取猪四肢密质骨，取材大小视临床实际手术需要而定，通常大小为1*1*0.5cm；其 余参考实施例1的方法进行，得到完全脱钙、脱细胞密质骨材料。

[0059]

Histological evaluation, quantitative detection of antigen components, and osteogenic repair capacity testing were performed on the completely decalcified and decellularized dense bone material obtained in this case. The results are shown in Figures 2, 4, 6, and 8.

对本例所得完全脱钙、脱细胞密质骨材料进行组织学评价、抗原成分定量检测和 成骨修复能力检测，结果如图2、4、6、8。

Figure 2, HE staining, shows that the extracellular matrix of the completely decalcified and decellularized cancellous bone material is intact, and the nuclear components are completely removed, with no cells or debris remaining. Figure 4, DAPI staining, further indicates that the nuclear components in the material are negative, and the antigenicity is completely removed. Figure 6, quantitative detection of DNA antigen components, shows that the DNA removal rate through decellularization can reach more than 95%. Figure 8, a rabbit tibia large-area bone defect repair experiment, shows that large-area bone defects were well repaired after using completely decalcified and decellularized cancellous bone material.

图2HE染色显示完全脱钙、脱细胞松质骨材料细胞 外基质保留完整，细胞核成分完全去除，无细胞及其碎片残留；图4DAPI染色进一步提示材 料中细胞核成分呈阴性，抗原性得到完全去除；图6的DNA抗原成分定量检测说明通过去细 胞DNA去除率可以达到95%以上；图8的兔子胫骨大面积骨缺损修复实验说明在使用了完全 脱钙、脱细胞松质骨材料后大面积大面积骨缺损得到了很好的修复。

[0060]

Example 3: Preparation and Study of Completely Decalcified and Decellularized Cancellous and Compact Bone Materials

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实施例3完全脱钙、脱细胞松质骨和密质骨材料的制备和研究

[0061]

Take cancellous bone from the iliac crest and compact bone from the limbs. In step (2), place the cancellous bone in 1000 ml of physiological saline buffer containing protease inhibitor (concentration of 5%, protease inhibitor content of 10 KIU/ml) and shake at 150 rpm for 8 hours at a constant temperature of 45°C. The rest of the process is carried out according to the method in Example 1 to obtain completely decalcified and decellularized cancellous and compact bone materials.

取猪髂骨松质骨和四肢密质骨，步骤(2)在1000ml含蛋白酶抑制剂的生理盐水缓 冲液(浓度为5%，蛋白酶抑制剂含量为10KIU/ml)中，恒温45°C摇床150rpm震荡8小时；其余 参考实施例1的方法进行，得到完全脱钙、脱细胞松质骨和密质骨材料。

[0062]

Example 4: Preparation and Study of Completely Decalcified and Decellularized Cancellous and Compact Bone Materials

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实施例4完全脱钙、脱细胞松质骨和密质骨材料的制备和研究

[0063]

Take cancellous bone from the iliac crest and compact bone from the limbs. In step (4), add 100 ml of penicillin and streptomycin mixed antibacterial solution to 1000 ml of PBS buffer containing TritonX (5% TritonX-200). Shake at 150 rpm for 3 hours at a constant temperature of 45°C. The rest of the process is carried out according to the method in Example 1 to obtain completely decalcified and decellularized cancellous and compact bone materials.

取猪髂骨松质骨和四肢密质骨，步骤(4)在1000ml含TritonX的PBS缓冲液(浓度 5%的TritonX-200)中，加入100ml青霉素和链霉素混合抗菌液，恒温45°C摇床150rpm震荡 3小时；其余参考实施例1的方法进行，得到完全脱钙、脱细胞松质骨和密质骨材料。

Example 5: Preparation and study of completely decalcified, decellularized cancellous bone and compact bone materials

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实施例5 完全脱钙、脱细胞松质骨和密质骨材料的制备和研究

[0064]

Take cancellous bone from the iliac crest and compact bone from the limbs. In step (5), add 100 ml of penicillin and streptomycin mixed antibacterial solution to 1000 ml of PBS buffer containing SDS (SDS concentration of 10%, mixed with 20 mmol of Tris), and shake at 150 rpm

for 2 hours at a constant temperature of 45°C. The rest of the process is carried out according to the method in Example 1 to obtain completely decalcified and decellularized cancellous bone and compact bone materials.

取猪髂骨松质骨和四肢密质骨，步骤(5)在1000ml含SDS的PBS缓冲液(SDS浓度为 10%，混合20mmol的Tris)中，加入100ml青霉素和链霉素混合抗菌液，恒温45°C摇床150rpm 震荡2小时；其余参考实施例1的方法进行，得到完全脱钙、脱细胞松质骨和密质骨材料。

[0065]

Example 6: Preparation and Study of Completely Decalcified and Decellularized Cancellous and Compact Bone Materials

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实施例6完全脱钙、脱细胞松质骨和密质骨材料的制备和研究

[0066]

Take cancellous bone from the iliac crest and compact bone from the limbs. In step (6), add 60 ml of a mixed antibacterial solution of penicillin and streptomycin to 300 ml of PBS buffer containing trypsin (trypsin concentration of 5%), and shake at 150 rpm for 12 hours at 37°C. The rest of the process is carried out according to the method of Example 1 to obtain completely decalcified and decellularized cancellous and compact bone materials.

取猪髂骨松质骨和四肢密质骨，步骤(6)在300ml含胰酶的PBS缓冲液(胰酶浓度为 5%)中，加入60ml青霉素和链霉素混合抗菌液，37°C摇床150rpm震荡12小时；其余参考实施 例1的方法进行，得到完全脱钙、脱细胞松质骨和密质骨材料。

[0067]

Example 7: Preparation and Study of Completely Decalcified and Decellularized Cancellous and Compact Bone Materials

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实施例7完全脱钙、脱细胞松质骨和密质骨材料的制备和研究

[0068]

Take cancellous bone from the iliac crest and compact bone from the limbs. In step (7), add 60 ml of penicillin and streptomycin mixed antibacterial solution to 300 ml of PBS buffer containing DNase (DNase concentration is 0.5 mg/ml), and shake at 150 rpm for 1 hour at 37° C. The rest of the process is carried out according to the method in Example 1 to obtain completely decalcified and decellularized cancellous and compact bone materials.

取猪髂骨松质骨和四肢密质骨，步骤(7)在300ml含DNA酶的PBS缓冲液中(DNA酶浓 度为0.5mg /ml)，加入60ml青霉素和链霉素混合抗菌液，37°C摇床150rpm震荡1小时；其余参 考实施例1的方法进行，得到完全脱钙、脱细胞松质骨和密质骨材料。

[0069]

Example 8: Preparation and Study of Completely Decalcified and Decellularized Cancellous and Compact Bone Materials

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实施例8完全脱钙、脱细胞松质骨和密质骨材料的制备和研究

[0070]

Take cancellous bone from the iliac crest and compact bone from the limbs. In step (3), degrease the bone by shaking at 150 rpm for 2 hours in 1000 ml of 70% ethanol solution at a constant temperature of 45 °C. The rest of the process is carried out according to the method in Example 1 to obtain completely decalcified and decellularized cancellous and compact bone materials.

取猪髂骨松质骨和四肢密质骨，步骤(3)在1000ml浓度70%的乙醇溶液中，恒温45 °C摇床150rpm震荡脱脂2小时；其余参考实施例1的方法进行，得到完全脱钙、脱细胞松质骨 和密质骨材料。

[0071]

Example 9: Preparation and Study of Completely Decalcified and Decellularized Cancellous and Compact Bone Materials

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实施例9完全脱钙、脱细胞松质骨和密质骨材料的制备和研究

[0072]

Take cancellous bone from the iliac crest and compact bone from the limbs. In step (8), treat the cancellous bone in 1000 ml of EDTA isotonic solution (concentration of 10%) with an ultrasonic machine with a power of 20 W for 24 hours. The rest are carried out according to the method of Example 1 to obtain completely decalcified and decellularized cancellous and compact bone materials.

取猪髂骨松质骨和四肢密质骨，步骤(8)在1000mlEDTA等渗液(浓度为10%)中， 以功率20W的超声波机器处理24小时；其余参考实施例1的方法进行，得到完全脱钙、脱细胞 松质骨和密质骨材料。

[0073]

Example 10: Preparation and Study of Completely Decalcified and Decellularized Cancellous and Compact Bone Materials

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实施例10完全脱钙、脱细胞松质骨和密质骨材料的制备和研究

[0074]

Take cancellous bone from the iliac crest and compact bone from the limbs. In step (8), treat the bone in 1000 ml of EDTA isotonic solution (concentration of 2%) with an ultrasonic machine with a power of 20 W for 24 hours. The rest of the process is carried out according to the method in Example 1 to obtain completely decalcified and decellularized cancellous and compact bone materials.

取猪髂骨松质骨和四肢密质骨，步骤(8)在1000mlEDTA等渗液(浓度为2%)中，以 功率20W的超声波机器处理24小时；其余参考实施例1的方法进行，得到完全脱钙、脱细胞松 质骨和密质骨材料。

[0075]

Example 11: Preparation and Study of Completely Decalcified and Decellularized Cancellous and Compact Bone Materials

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实施例11完全脱钙、脱细胞松质骨和密质骨材料的制备和研究

[0076]

Take cancellous bone from the iliac crest and compact bone from the limbs. In step (8), treat the bone in 1000ml of EDTA isotonic solution (concentration of 2%) with an ultrasonic machine with a power of 200W for 2 hours. The rest of the process is carried out according to the method in Example 1 to obtain completely decalcified and decellularized cancellous and compact bone materials.

取猪髂骨松质骨和四肢密质骨，步骤(8)在1000mlEDTA等渗液(浓度为2%)中，以 功率200W的超声波机器处理2小时；其余参考实施例1的方法进行，得到完全脱钙、脱细胞松 质骨和密质骨材料。

[0077]

Example 12: Preparation and Study of Completely Decalcified and Decellularized Cancellous and Compact Bone Materials

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实施例12完全脱钙、脱细胞松质骨和密质骨材料的制备和研究

[0078]

Take cancellous bone from the iliac crest and compact bone from the limbs. In step (8), treat the cancellous bone in 1000 ml of EDTA isotonic solution (concentration of 10%) with an

ultrasonic machine with a power of 20 W for 2 hours. The rest are carried out according to the method of Example 1 to obtain completely decalcified and decellularized cancellous and compact bone materials.

取猪髂骨松质骨和四肢密质骨，步骤(8)在1000mlEDTA等渗液(浓度为10%)中， 以功率20W的超声波机器处理2小时；其余参考实施例1的方法进行，得到完全脱钙、脱细胞 松质骨和密质骨材料。

[0079]

The completely decalcified, decellularized cancellous bone and compact bone materials obtained in Examples 3-12 were subjected to histological evaluation, quantitative detection of antigen components, and osteogenic repair capacity testing, respectively. The results were similar to those in Examples 1 and 2, indicating that completely decalcified, decellularized cancellous bone and compact bone can be obtained through this invention. Histological evaluation, quantitative detection of antigen components, and osteogenic repair capacity testing all showed that the material had completely removed cellular components and had no obvious antigen component residues. When applied to the repair of bone defects in large animal models such as rabbits, it can achieve very good bone repair and regeneration effects.

对实施例3-12所得完全脱钙、脱细胞松质骨和密质骨材料分别进行组织学评价、 抗原成分定量检测和成骨修复能力检测，结果与实施例1和2类似，这表明通过本发明可获 得完全脱钙、脱细胞松质骨

和密质骨，组织学评价、抗原成分定量检测和成骨修复能力检测 均说明材料已经完全去除细胞成分，无明显抗原成分残留；将其运用于兔子一类的大动物 骨缺损模型修复中可以达到很好的骨修复再生效果。

Completely decalcified, decellularized cancellous and compact bone materials can be used as safe, reliable, effective, and rapid bone-forming materials for patients with bone defects and those undergoing bone transplantation in clinical practice.

完全脱钙、脱细胞松质骨和密质骨材 料可以作为临床上骨缺损、骨移植等病人的安全可靠、有效、快速成骨材料。