Notice

DESCRIPTION CN113577391A

A method for preparing epiphyseal cartilage combined with decellularized bone material derived from natural tissue

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一种天然组织来源的骺软骨联合骨脱细胞材料的制备方法

[0001]

Technical Field

技术领域

[n0001]

This invention relates to the field of biomaterials technology for tissue or organ repair and regeneration, and more specifically to a method for preparing epiphyseal cartilage combined with decellularized bone material derived from natural tissues.

本发明涉及用于组织或器官修复与再生的生物材料技术领域，更具体的说是涉及一种天然组织来源的骺软骨联合骨脱细胞材料的制备方法。

[0003]

Background Technology

背景技术

[n0002]

As China gradually enters an aging society, diseases such as osteoarthritis and cartilage damage and defects caused by strenuous activity are increasingly affecting human health, imposing a heavy economic and physical burden on individuals, and creating a heavy social burden. These are pressing problems that need to be solved in orthopedic clinical practice.

目前随着中国逐步进入老龄化社会，骨关节炎及剧烈活动引起的软骨损伤及软骨缺损等疾病日益影响人类健康，对个人造成沉重的经济及身心负担，并造成了沉重社会负担，是骨科临床亟待解决的难题。

However, current traditional solutions to the above problems, such as arthroscopic repair surgery and joint replacement surgery, still have many issues, such as poor repair results and huge surgical trauma.

而针对上述难题，目前传统的解决方案包括关节镜修复手术、骨关节置换术等仍存在诸多问题，如修复效果欠佳、手术创伤巨大等问题。

In recent years, the use of biomaterials to repair cartilage tissue defects has received widespread attention. However, in addition to potential issues such as poor biocompatibility and insufficient cartilage repair and regeneration, current biomaterials also have the problem of focusing solely on hyaline cartilage as the repair target.

近年来采用生物材料修复软骨组织缺损受到了广泛关注，但目前的生物材料除了可能存在的生物相容性不良和软骨修复再生不足，还存在着修复目标仅聚焦于单纯透明软骨的问题。

As is well known, osteoarthritis and cartilage damage not only affect hyaline cartilage itself, but also the subchondral bone. Therefore, in the treatment of osteoarthritis and cartilage

defects, it is necessary to comprehensively repair both hyaline cartilage and subchondral bone. The development of biomaterials that promote the regeneration of cartilage and bone has become a bottleneck that urgently needs to be overcome in the treatment of osteoarthritis and cartilage defects.

众所周知，骨关节炎及软骨的损伤不仅累及透明软骨自身，还累及软骨下骨，因此在对骨关节炎和软骨缺损修复治疗中，需要综合修复透明软骨及软骨下骨。 研发具有促进软骨联合骨再生的生物材料成为了当前治疗骨关节炎及软骨缺损亟需突破的瓶颈。

[n0003]

The epiphyseal cartilage is located at both ends of long bones, where secondary ossification centers appear.

骺软骨在长骨的两端，二次骨化中心即在其中出现。

The middle layer of epiphyseal cartilage surrounding the secondary ossification center. In normal organisms, the epiphyseal cartilage eventually differentiates into bone tissue, leading to epiphyseal closure. However, some studies have found that the epiphyseal cartilage stores a layer of cartilage germ cells, which has the potential to differentiate into cartilage. Therefore, the epiphyseal symphysis bone has the ability to repair defects in the symphysis bone.

环绕在二次骨化中心周围的骺软骨中间层，在正常生物体中，骺软骨最终分化成骨组织使得骨骺闭。 但有研究发现，骺软骨贮备着一层软骨胚种细胞带，具有分化为软骨的潜力，因此骺软骨联合骨存在修复软骨联合骨缺损的能力。

[n0004]

Extracellular matrix derived from natural tissues can promote cell adhesion, growth, migration, and guide cell differentiation into bone tissue, making it a key focus of bone tissue engineering research in recent years.

天然组织来源的细胞外基质可以促进细胞粘附、生长、迁移并引导细胞向骨组织分化，是近年来骨组织工程研究的重点。

Natural decellularized tissue materials possess unique biocompatibility due to their high similarity to biological organisms in structure and composition, thus exhibiting excellent tissue repair effects. The use of decellularization technology to prepare extracellular scaffolds provides a feasible approach for the preparation of tissue repair and regeneration materials. Currently, there are few reports on biomaterials that can be used to repair synostosis cartilage, and there are no reports on the repair of osteoarthritis and cartilage defects using synostosis cartilage.

天然组织脱细胞材料因其结构和组成成分与生物机体高度相似而具有独特的生物相容性，因此表现出优异的组织修复效果。 运用脱细胞技术制备细胞外支架为组织修复再生材料的制备提供可行思路。而目前能用于修复软骨联合骨的生物材料较少见报道，而骺软骨联合骨修复骨关节炎及软骨缺损则未见报道。

[n0005]

Therefore, providing a method for preparing epiphyseal cartilage combined with decellularized bone material from natural tissue sources is a problem that urgently needs to be solved by those skilled in the art.

因此，提供一种天然组织来源的骺软骨联合骨脱细胞材料的制备方法是本领域技术人员亟需解决的问题。

[0008]

Summary of the Invention

发明内容

[n0006]

In view of this, the present invention provides a method for preparing epiphyseal cartilage combined with decellularized bone material from natural tissue. The prepared epiphyseal cartilage combined with decellularized bone material is a biomaterial with good material properties, excellent biocompatibility and excellent osteocartilage repair effect, providing a new material and approach for repairing cartilage defects and treating osteoarthritis.

有鉴于此，本发明提供了一种天然组织来源的骺软骨联合骨脱细胞材料的制备方法，制备得到的骺软骨联合骨脱细胞材料是一种具有良好材料特性、优越的生物相容性和优异的骨软骨修复效果的生物材料，为修复软骨缺损及治疗骨关节炎提供一种新的材料和途径。

[n0007]

To achieve the above objectives, the present invention adopts the following technical solution:

为了实现上述目的，本发明采用如下技术方案：

[n0008]

A method for preparing epiphyseal cartilage combined with decellularized bone material derived from natural tissues, the specific steps of which are as follows:

一种天然组织来源的骺软骨联合骨脱细胞材料的制备方法，具体步骤如下：

[n0009]

(1) Take fresh femoral distal epiphyseal cartilage fusion bone tissue from 3-4 month old Large White pigs (male or female), and rinse it repeatedly with sterile PBS 5 times at 4°C for 10 minutes each time to remove blood and tissue fluid from the material;

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(1)取新鲜3-4月龄大白猪(雌雄不限)股骨远端骺软骨联合骨组织，在4°C低温环境下用无菌PBS反复漂洗5次，每次10min；去除材料上的血液、组织液；

[n0010]

(2) In an organic solvent, place the mixture in a constant temperature shaker at 25°C and 60 rpm for 2-6 hours to degrease; then rinse with sterile PBS for 12 hours; the organic solvent is a 50-70% ethanol solution or a 20-40% acetone solution.

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(2)在有机溶剂中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂2-6h；后用无菌PBS冲洗12h；所述有机溶剂为质量浓度为50-70%乙醇溶液或20-40%丙酮溶液；

[n0011]

(3) After placing it in liquid nitrogen for 30 min, quickly remove it and place it in a 37°C water bath for 30 min; repeat the above process 3 times, and finally rinse twice with sterile PBS for 5 min each time;

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(3)置于液氮30min后，再迅速取出放置于37°C水浴30min；上述过程重复3次，最后用无菌PBS漂洗2次，每次5min；

[n0012]

(4) Add the mixed antibacterial solution to PBS buffer containing Triton X-100 with a volume concentration of 1-2%, place it in a constant temperature shaker at 25°C and 100rpm for 24-72h; then rinse with sterile PBS for 12h.

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(4)在体积浓度为1-2%的含Triton X-100的PBS缓冲液中加入混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡24-72h；再用无菌PBS冲洗12h；

[n0013]

(5) Add the mixed antibacterial solution to a PBS buffer containing sodium dodecyl ether sulfate (SLES) with a mass concentration of 2-5%, and place it in a constant temperature shaker at 25°C and 100 rpm for 12-48 hours; then rinse with sterile PBS for 12 hours.

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(5)在质量浓度为2-5%的含十二烷基醚硫酸钠磺酸(SLES)的PBS缓冲液中加入混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡12-48h；再用无菌PBS冲洗12h；

[n0014]

(6) Add the mixed antibacterial solution to PBS buffer containing 1-2% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HT HOPS), and place it in a constant temperature shaker at 25°C and 100 rpm for 6-12 hours; then rinse with sterile PBS for 12 hours.

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(6)在质量浓度为1-2%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HT HOPS)的PBS缓冲液中加入混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡6-12h；再用无菌PBS冲洗12h；

[n0015]

(7) Add the mixed antibacterial solution to PBS buffer containing 0.01 mg/ml DNase I and incubate at 37°C for 6-12 hours.

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(7)在含0.01mg/ml DNase Ⅰ的PBS缓冲液中，加入混合抗菌溶液，37°C恒温水浴6-12h；

[n0016]

(8) Place the ethanol with a mass concentration of 50-70% in a constant temperature shaker at 25°C and 120rpm for 24-48h.

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(8)在质量浓度为50-70%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡24-48h；

[n0017]

(9) Add a mixed antibacterial solution to sterile physiological saline, place it in a constant temperature shaker at 37°C and 120 rpm for 12 hours, and repeat 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

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(9)在无菌生理盐水中，加入混合抗菌溶液，置于温度为37°C、转速为120rpm的恒温摇床中震荡12h，重复3次；得到天然组织来源的骺软骨联合骨脱细胞材料；

[n0018]

In steps (4)-(7), the concentrations of penicillin and streptomycin in the mixed antibacterial solution are 100 U/ml and 100 μg/ml, respectively; the volume ratio of penicillin to streptomycin is 1:1; and the volume ratio of PBS buffer to the mixed antibacterial solution is 10:1.

步骤(4)-(7)中，所述混合抗菌溶液中青霉素和链霉素的浓度分别为100U/ml，100μg/ml；青霉素和链霉素的体积比为1:1；PBS缓冲液和混合抗菌溶液的体积比为10:1；

[n0019]

In step (9), the concentrations of penicillin and streptomycin in the mixed antibacterial solution are 100 U/ml and 100 μg/ml, respectively; the volume ratio of penicillin to streptomycin is 1:1; and the volume ratio of sterile saline to the mixed antibacterial solution is 10:1.

步骤(9)中，所述混合抗菌溶液中青霉素和链霉素的浓度分别为100U/ml，100μg/ml；青霉素和链霉素的体积比为1:1；无菌生理盐水和混合抗菌溶液的体积比为10:1。

[n0020]

All of the above steps should be performed in a relatively sterile environment.

上述步骤均尽可能保持相对无菌。

[n0021]

This invention addresses the problems existing in current methods for preparing materials for cartilage-bone repair and regeneration by establishing a method for preparing decellularized

epiphyseal cartilage-bone material derived from natural tissue. This method involves repeatedly washing, defatting, repeatedly freezing and thawing, and detoxifying the distal femoral epiphyseal cartilage-bone tissue of young Large White pigs with PBS buffer containing Triton X-100, SLES, HT HOPS, DNase I, and physiological saline to obtain the decellularized epiphyseal cartilage-bone material.

本发明针对目前用于软骨联合骨修复与再生材料的制备方法存在的问题，建立了一种天然组织来源的骺软骨联合骨脱细胞材料的制备方法，该方法将幼年大白猪股骨远端骺软骨联合骨组织经过反复漂洗、脱脂、反复冻融、含Triton X-100的PBS缓冲液、含SLES的PBS缓冲液、含HT HOPS的PBS缓冲液、含DNase Ⅰ的PBS缓冲液和生理盐水脱毒处理后，获得骺软骨联合骨脱细胞材料。

[n0022]

As can be seen from the above technical solution, compared with the prior art, the present invention discloses a method for preparing epiphyseal cartilage combined with decellularized bone material from natural tissue, which has the following beneficial effects:

经由上述的技术方案可知，与现有技术相比，本发明公开提供了一种天然组织来源的骺软骨联合骨脱细胞材料的制备方法，具有以下有益效果：

[n0023]

(1) This invention is the first to achieve the preparation of decellularized material of epiphyseal cartilage synostosis bone, and uses SLES and HT HOPS to replace sodium dodecyl sulfate (SDS). SLES and HT HOPS are both detergents, but they are milder and have a remarkable decellularization effect than SDS. They can completely remove cells and their cell contents from the material while preserving the integrity of the original natural ECM components and structure to a greater extent. Moreover, the structure of epiphyseal cartilage synostosis bone is very similar to that of normal cartilage synostosis bone, and can simulate the composition and structure of normal cartilage synostosis bone tissue to the greatest extent.

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(1)本发明首次实现对骺软骨联合骨脱细胞材料的制备，并采用SLES及HT HOPS替代十二烷基硫酸钠(SDS)，SLES及HT HOPS同为去垢剂，但相对于SDS更温和且脱细胞效果可观，能在完全去除材料中细胞及其细胞内容物的同时，更大程度地保留原有的天然ECM成分和结构的完整性，且骺软骨联合骨结构与正常软骨联合骨的结构十分相似，能最大程度模拟正常软骨联合骨组织的成分和结构。

[n0024]

(2) The material of the present invention, after being decellularized and repeatedly rinsed with ethanol and sterile saline for detoxification, does not have cytotoxicity and immunogenicity, and has good biocompatibility and excellent cartilage-bone defect repair effect.

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(2)本发明材料通过脱细胞后，再用乙醇及无菌生理盐水反复漂洗进行脱毒后，不具有细胞毒性和免疫原性，并拥有良好的生物相容性和优异的软骨联合骨缺损修复效果。

[n0025]

(3) The material of this invention is derived from the femur of young large white pigs. The raw materials are widely available and can be used for mass production.

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(3)本发明材料源于幼年大白猪股骨，原材料来源广泛，可用于批量生产。

[n0026]

(4) The present invention can be a personalized epiphyseal cartilage-associated bone biomaterial with customizable size and dimensions to meet the complex and diverse clinical needs for repairing cartilage-associated bone.

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(4)本发明可为大小及尺寸可定制的个体化骺软骨联合骨生物材料，以用于满足临床上复杂多样的修复软骨联合骨的需要。

[0030]

Attached Figure Description

附图说明

[n0027]

To more clearly illustrate the technical solutions in the embodiments of the present invention or the prior art, the drawings used in the description of the embodiments or the prior art will be briefly introduced below. Obviously, the drawings described below are only embodiments of the present invention. For those skilled in the art, other drawings can be obtained based on the provided drawings without creative effort.

为了更清楚地说明本发明实施例或现有技术中的技术方案，下面将对实施例或现有技术描述中所需要使用的附图作简单地介绍，显而易见地，下面描述中的附图仅仅是本发明的实施例，对于本领域普通技术人员来讲，在不付出创造性劳动的前提下，还可以根据提供的附图获得其他的附图。

[n0028]

Figure 1 is a schematic diagram of the epiphyseal cartilage combined bone harvesting site of the present invention;

图1附图为本发明骺软骨联合骨取材部位示意图；

[n0029]

The left image shows a gross view of the distal femoral epiphyseal cartilage plate, with the sampling site indicated within the box; the right image shows the sampling site stained with hematoxylin and eosin (HE), revealing the △ area as the epiphyseal cartilage region.

其中，左图为股骨远端骨骺软骨板大体图，方框内为取材部位；右图为将取材部位进行HE染色，显示△区域为骺软骨区域；

[n0030]

Figure 2 shows the DNA quantification detection results of different regions in the decellularized epiphyseal cartilage and bone decellularized material before and after decellularization according to the present invention; ***: p < 0.001;

图2附图为本发明脱细胞前后骺软骨联合骨脱细胞材料各区域DNA定量检测图；***：p＜0.001；

[n0031]

Figure 3 is an evaluation diagram of the decellularization efficacy of the combined epiphyseal cartilage and bone decellularization material of the present invention; wherein, the second column is a macroscopic view of the third, fourth and fifth columns; the scale bar is 100 μm;

图3附图为本发明脱细胞前后骺软骨联合骨脱细胞材料脱细胞效能评估图；其中，第2列图为第3、4、5列图的宏观图；比例尺大小为100μm；

[n0032]

Figure 4 is a qualitative evaluation diagram of the retention of the main components of the decellularized epiphyseal cartilage combined with bone decellularization material before and after decellularization according to the present invention; wherein, the first column of the figure is a macroscopic view of the second, third and fourth columns of the figure; the scale bar is 100μm;

图4附图为本发明脱细胞前后骺软骨联合骨脱细胞材料主要成分保留定性评估图；其中，第1列图为第2、3、4列图的宏观图；比例尺大小为100μm；

[n0033]

Figure 5 shows the quantitative evaluation of the main components retained in the decellularized epiphyseal cartilage combined with bone decellularization material of the present invention before and after decellularization; the left figure is a bar chart for quantitative evaluation of collagen; the right figure is a bar chart for quantitative evaluation of glycosaminoglycans (GAG); ns: $p>0.05$; *: $p<0.05$;

图5附图为本发明脱细胞前后骺软骨联合骨脱细胞材料主要成分保留定量评估图；其中，左图为胶原蛋白定量评估条形图；右图为糖胺聚糖(GAG)定量评估条形图；ns：p＞0.05；*：p＜0.05；

[n0034]

Figure 6 shows the three-dimensional structure preservation evaluation diagram of the decellularized epiphyseal cartilage combined with bone decellularization material of the present invention;

图6附图为本发明脱细胞前后骺软骨联合骨脱细胞材料三维结构保留评估图；

[n0035]

Figure 7 is a uniaxial compression curve diagram of the normal group of the present invention;

图7附图为本发明正常组的单轴压缩曲线图；

[n0036]

Figure 8 is a uniaxial compression curve of the decellularized group of the present invention;

图8附图为本发明脱细胞组的单轴压缩曲线图；

[n0037]

Figure 9 is a statistical graph of the elastic modulus of the present invention; ns: p > 0.05;

图9附图为本发明弹性模量统计图；ns：p＞0.05；

[n0038]

Figure 10 is a statistical diagram of the relative elastic limit of the present invention; *: p < 0.05;

图10附图为本发明相对弹性极限统计图；*：p＜0.05；

[n0039]

Figure 11 shows the cytotoxicity evaluation of the decellularized epiphyseal cartilage combined with decellularized bone material of the present invention; ns: p>0.05;

图11附图为本发明脱细胞骺软骨联合骨脱细胞材料细胞毒性评估图；ns：p＞0.05；

[n0040]

Figure 12 shows the cytotoxicity and cell adhesion evaluation of the decellularized epiphyseal cartilage combined with decellularized bone material of the present invention; where a, b, and c represent cells in the epiphyseal cartilage, junctional zone, and cancellous bone zone, respectively, to demonstrate the adhesiveness of the scaffold and its ability to promote the proliferation and migration of target cells; the images within the boxes are low-power images, and the images outside the boxes are high-power images of selected specific targets within the low-power field; the scale bar is 60 μm.

图12附图为本发明脱细胞骺软骨联合骨脱细胞材料细胞毒性、细胞粘附评估图；其中，a、b、c分别表示在骺软骨，交界区和松质骨区中细胞，以表明支架的可粘附性和促进目标细胞的增殖迁移；方框内的图为低倍镜图，方框外的为低倍镜中选取特定目标的高倍镜图；比例尺大小为60μm；

[n0041]

Figure 13 shows the immunogenicity evaluation diagram of the decellularized epiphyseal cartilage combined with decellularized bone material of the present invention;

图13附图为本发明脱细胞骺软骨联合骨脱细胞材料免疫原性评估图；

[n0042]

The first column of images is a general view of the embedded structure, while the second and third columns are magnified views of the epiphyseal cartilage and bone in the first column, respectively; the scale bar is 100 μm.

其中，第一列图“大体”为包埋的整体观图片，第2列和第3列分别是第1列图中骺软骨和骨的放大图；比例尺大小为100μm；

[n0043]

Figure 14 shows the in vitro osteogenic and chondrogenic induction evaluation of the decellularized epiphyseal cartilage combined with decellularized bone material of the present invention; ns: p>0.05; *: p<0.05; **: p<0.01; the left side of each bar chart is the blank group, and the right side is the experimental group;

图14附图为本发明脱细胞骺软骨联合骨脱细胞材料体外成骨、成软骨诱导评估图；ns：p＞0.05；*：p＜0.05；**：p＜0.01；每组柱形图左侧为空白组，右侧为实验组；

[n0044]

Figure 15 shows the in vivo osteogenic and chondrogenic induction tissue evaluation of the decellularized epiphyseal cartilage combined with decellularized bone material of the present invention; the scale bar is 1 mm.

图15附图为本发明脱细胞骺软骨联合骨脱细胞材料在体成骨、成软骨诱导组织评估图；比例尺大小为1mm；

[n0045]

Figure 16 shows the proportion of cartilage in the cartilage region of the present invention; *: p < 0.05; ***: p < 0.001;

图16附图为本发明软骨区域内软骨所占比例；*：p＜0.05；***：p＜0.001；

[n0046]

Figure 17 shows the proportion of cartilage in the subchondral bone region of the present invention; ns: p > 0.05; ***: p < 0.001;

图17附图为本发明软骨下骨区域内软骨所占比例；ns：p＞0.05；***：p＜0.001；

[n0047]

Figure 18 shows the proportion of bone in the subchondral bone region of the present invention; ***: p < 0.001;

图18附图为本发明软骨下骨区域内骨质所占比例；***：p＜0.001；。

[0052]

Detailed Implementation

具体实施方式

[n0048]

The technical solutions of the present invention will be clearly and completely described below with reference to the accompanying drawings of the embodiments of the present invention. Obviously, the described embodiments are only some embodiments of the present invention, and not all embodiments.

下面将结合本发明实施例中的附图，对本发明实施例中的技术方案进行清楚、完整地描述，显然，所描述的实施例仅仅是本发明一部分实施例，而不是全部的实施例。

Based on the embodiments of the present invention, all other embodiments obtained by those skilled in the art without creative effort are within the scope of protection of the present invention.

基于本发明中的实施例，本领域普通技术人员在没有做出创造性劳动前提下所获得的所有其他实施例，都属于本发明保护的范围。

[n0049]

Example 1

实施例1

[n0050]

A method for preparing epiphyseal cartilage combined with decellularized bone material derived from natural tissues, the specific steps of which are as follows:

一种天然组织来源的骺软骨联合骨脱细胞材料的制备方法，具体步骤如下：

[n0051]

(1) Material collection: Take fresh femoral distal epiphyseal fusion bone from 3-month-old juvenile Large White pigs and rinse it 5 times with sterile PBS buffer at 4°C for 10 minutes each

time to remove impurities such as hair, muscle, fascia, blood and tissue fluid from the surface; the size of the material is determined according to actual needs, usually a cylinder with a diameter of 5 mm and a height of 5 mm is taken.

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(1)取材：取新鲜3月龄幼年大白猪股骨远端骺软骨联合骨，在4℃低温环境下用无菌PBS缓冲液漂洗5次，每次10min，去除表面的毛发、肌肉、筋膜、血液和组织液等杂质；取材大小视实际需求确定，通常大小取直径为5mm，高度为5mm的圆柱体；

[n0052]

(2) Decellularization process:

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(2)脱细胞流程：

[n0053]

① Place the solution in 500ml of organic solvent solution (20% acetone solution) in a constant temperature shaker at 25°C and 60rpm for 4 hours to degrease, and then rinse with sterile PBS for 12 hours.

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①、在500ml有机溶剂溶液(20%丙酮溶液)中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂4h，后用无菌PBS冲洗12h；

[n0054]

② After drying the material with sterile gauze, place it in liquid nitrogen for 30 minutes, then quickly remove it and place it in a 37°C water bath for 30 minutes; repeat 3 times; finally, rinse twice with sterile PBS for 5 minutes each time.

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②、用无菌纱布将材料擦干后，置于液氮30min后，再迅速取出放置于37°C水浴30min；重复3次；最后用无菌PBS漂洗2次，每次5min；

[n0055]

③ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% Triton-X100. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 48 h; then rinse with sterile PBS for 12 h.

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③、在500ml含有体积浓度为1%Triton-X100的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡48h；再用无菌PBS冲洗12h；

[n0056]

④ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2% SLES. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 36 h; then rinse with sterile PBS for 12 h.

---

④、在500ml含有质量浓度为2%SLES的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡36h；再用无菌PBS冲洗12h；

[n0057]

⑤ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HTHOPS). Place the solution in a constant temperature shaker at 25°C and 100 rpm for 6 h; then rinse with sterile PBS for 12 h.

---

⑤、在500ml含有质量浓度为1%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HTHOPS)的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡6h；再用无菌PBS冲洗12h；

[n0058]

⑥ Add 10 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 100 ml of PBS buffer containing 0.01 mg/ml DNase I, and place it in a water bath at 37°C for 12 h.

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⑥、在100ml含浓度为0.01mg/ml的DNase Ⅰ的PBS缓冲液中，加入10ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温37°C水浴箱中12h；

[n0059]

⑦ Place 500 ml of 70% ethanol in a constant temperature shaker at 25°C and 120 rpm for 24 hours.

---

⑦、在500ml质量浓度为70%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡24h；

[n0060]

⑧. Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of sterile physiological

saline. Place the solution in a constant temperature shaker at 37°C and 120 rpm for 12 h. Repeat this process 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

---

⑧、在500ml无菌生理盐水中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于温度为37°C、转速为120rpm的恒温摇床中震荡12h，重复3次，即得到天然组织来源的骺软骨联合骨脱细胞材料。

[n0061]

Using the epiphyseal cartilage combined with decellularized bone material prepared in Example 1 as a template, a series of tests and experiments were conducted, as follows:

以实施例1制备出的骺软骨联合骨脱细胞材料作为模板进行一系列检测和实验，具体如下：

[n0062]

Figure 1 shows a schematic diagram of the epiphyseal cartilage combined bone sampling site. The left image shows a gross view of the distal femoral epiphyseal cartilage plate, with the sampling site within the box. The right image shows the site after HE staining, with the △ area indicating the epiphyseal cartilage region.

骺软骨联合骨取材部位示意图见图1；图1显示，左图为股骨远端骨骺软骨板大体图，方框内为取材部位；右图为将该部位进行HE染色，显示△区域为骺软骨区域。

[n0063]

Figure 2 shows the quantitative detection of DNA in different regions of the combined epiphyseal cartilage and bone decellularization material before and after decellularization. DNA was extracted from the cancellous bone and epiphyseal regions of the combined epiphyseal cartilage and bone decellularization material before and after decellularization using a DNA genome extraction kit, and the corresponding OD values were measured. The DNA content in each region was also determined.

图2为脱细胞前后骺软骨联合骨脱细胞材料各区域DNA定量检测图；采用DNA基因组提取试剂盒提取脱细胞前后骺软骨联合骨脱细胞材料中松质骨和骨骺区域DNA并测定相应OD值，测定各区域DNA含量。

The results showed that the DNA in the decellularized epiphyseal cartilage and bone decellularized material was reduced by more than 95% compared with the pre-decellularized scaffold, with no obvious DNA residue.

结果显示脱细胞后骺软骨联合骨脱细胞材料细胞中DNA较去细胞前支架减少95%以上，无明显DNA残留。

(***: p < 0.001).

(***：p＜0.001)。

[n0064]

Figure 3 shows the decellularization efficiency evaluation of the epiphyseal cartilage combined bone decellularization material before and after decellularization; the gross images of the epiphyseal cartilage combined bone decellularization material before and after decellularization show that the scaffold changed from pink to milky white after decellularization; the HE staining image shows that there are no obvious cells and cell contents remaining on the scaffold after decellularization; the DAPI staining image shows that there are no obvious cell nuclei and cell fragments remaining on the scaffold after decellularization.

图3为脱细胞前后骺软骨联合骨脱细胞材料脱细胞效能评估图；脱细胞前后骺软骨联合骨脱细胞材料大体图显示脱细胞后支架由粉红色变成乳白色；HE染色图显示脱细胞后支架无明显细胞及细胞内容物残留；DAPI染色图显示脱下表后支架无明显细胞核及细胞核碎片残留。

In summary, this indicates that the epiphyseal cartilage combined with decellularized bone material has a good decellularization effect, and the scaffold has no obvious cells or residual contents.

综上所述，表明骺软骨联合骨脱细胞材料脱细胞效果良好，支架已无明显细胞及其内容无残留。

[n0065]

Figure 4 shows the qualitative assessment of the retention of the main components of the epiphyseal cartilage and bone decellularized materials before and after decellularization; the first and second rows are Masson trichrome staining images, which show that the collagen component in the epiphyseal cartilage and bone decellularized materials before and after decellularization is well preserved; the third and fourth rows are Safranin O fast green staining images, which show that the epiphyseal cartilage glycosaminoglycans are lost more after decellularization, while the glycosaminoglycans in the cancellous bone are basically well preserved.

图4为脱细胞前后骺软骨联合骨脱细胞材料主要成分保留定性评估图；第一和第二行图为Masson三色染色图，结果表明脱细胞前后骺软骨联合骨脱细胞材料中胶原成分保留完好；第三和第四行图为番红O快绿染色，表明脱细胞后骺软骨糖胺聚糖丢失较多，而松质骨部分糖胺聚糖基本保留完好。

[n0066]

Figure 5 shows the quantitative assessment of the retention of major components in the epiphyseal cartilage and bone decellularization materials before and after decellularization; the left figure is a bar chart for quantitative assessment of collagen, showing that the collagen component in the epiphyseal cartilage and bone decellularization materials is well preserved after decellularization; the right figure is a bar chart for quantitative assessment of glycosaminoglycans (GAG), showing that the GAG content in the decellularized epiphyseal cartilage is significantly lower than that in the normal group, and the GAG in the cancellous bone is well preserved after decellularization.

图5为脱细胞前后骺软骨联合骨脱细胞材料主要成分保留定量评估图；左图为胶原蛋白定量评估条形图，结果显示脱细胞后骺软骨联合骨脱细胞材料中胶原蛋白成分保留完好；右图为糖胺聚糖(GAG)定量评估条形图，结果显示脱细胞骺软骨GAG含量较正常组有明显下降，脱细胞后松质骨部分GAG保留完好。

[n0067]

Figure 6 shows the evaluation of the preservation of the three-dimensional structure of the epiphyseal cartilage combined with bone decellularization material before and after decellularization. The three-dimensional structure of the surface of the epiphyseal cartilage combined with bone decellularization material before and after decellularization was

evaluated by scanning electron microscopy (SEM). The results showed that the three-dimensional structure of each part of the scaffold surface was not significantly damaged, and the porosity of the scaffold was significantly increased after decellularization.

图6为脱细胞前后骺软骨联合骨脱细胞材料三维结构保留评估图；通过扫描电镜(SEM)对脱细胞前后骺软骨联合骨脱细胞材料表面三维结构进行评估，结果显示支架各部分表面的三维结构未受到明显破坏，且脱细胞后支架的孔隙率明显增大。

[n0068]

Figures 7-10 show the quantitative evaluation of the mechanical retention of decellularized epiphyseal cartilage combined with bone before and after decellularization. The mechanical properties of the decellularized epiphyseal cartilage combined with bone before and after decellularization were determined by uniaxial compression test. The results showed that the mechanical properties of the decellularized epiphyseal cartilage combined with bone were well preserved after decellularization.

图7-图10为脱细胞前后骺软骨联合骨脱细胞材料力学保留定量评估图；采用单轴压缩试验对脱细胞前后骺软骨联合骨脱细胞材料力学性能进行测定，结果表明脱细胞后骺软骨联合骨脱细胞材料力学性能保留良好。

[n0069]

Figure 11 shows the cytotoxicity assessment of decellularized epiphyseal cartilage combined with decellularized bone material. The extract of decellularized epiphyseal cartilage combined with decellularized bone material was extracted using the CCK-8 method, and the concentration of cell metabolites (OD values) was measured after dilution at different ratios. The OD values were compared with the OD values of cell metabolite concentrations measured after culture in ordinary culture medium. The results showed that decellularized epiphyseal cartilage combined with decellularized bone material had no significant cytotoxicity.

图11为脱细胞骺软骨联合骨脱细胞材料细胞毒性评估图；采用cck-8法提取脱细胞骺软骨联合骨脱细胞材料浸提液，并稀释不同倍数后测定细胞代谢产物浓度OD值，上述OD值与普通培养基培养后测定细胞代谢产物浓度的OD值对比，结果表明脱细胞骺软骨联合骨脱细胞材料无明显细胞毒性。

[n0070]

Figure 12 shows the cytotoxicity and cell adhesion assessment of decellularized epiphyseal cartilage combined with decellularized bone material. One week after mouse bone mesenchymal stem cells (BMSCs) were seeded on decellularized epiphyseal cartilage combined with decellularized bone material, it can be seen that BMSCs can adhere normally

to the scaffold and migrate into the cancellous bone region inside the scaffold (Figure C), indicating that the scaffold is non-cytotoxic and can act as a medium for normal cell adhesion and migration.

图12为脱细胞骺软骨联合骨脱细胞材料细胞毒性、细胞粘附评估图；用小鼠骨间充质干细胞(BMSCs)种植在脱细胞骺软骨联合骨脱细胞材料上1周后，可见BMSCs可正常粘附于支架上，并可向支架内部松质骨区域迁移(图C)，表明该支架无细胞毒性，且能作为介质让细胞正常粘附、迁移。

[n0071]

Figure 13 shows the immunogenicity assessment of decellularized epiphyseal cartilage combined with decellularized bone material. The immunogenicity of the scaffold was assessed by implanting decellularized epiphyseal cartilage combined with decellularized bone material into the subcutaneous tissue of the back skin of rats for 2 and 4 weeks. The results showed that there was a certain degree of inflammatory response at 2 weeks; however, the inflammatory response was significantly reduced at 4 weeks, and signs of scaffold degradation were visible.

图13为脱细胞骺软骨联合骨脱细胞材料免疫原性评估图；将脱细胞骺软骨联合骨脱细胞材料埋植于大鼠背部皮肤皮下组织2、4周以评估支架免疫原性，结果显示2周时存在一定程度的炎症反应；但4周时炎症反应明显减弱，且可见支架出现降解迹象。

[n0072]

Figure 14 shows the in vitro osteogenic and chondrogenic induction evaluation of decellularized epiphyseal cartilage combined with decellularized bone material. Mouse bone mesenchymal stem cells were seeded on decellularized epiphyseal cartilage combined with decellularized bone material for 3, 7, and 14 days. Cells were collected and RNA was extracted. After PCR experiments, the relative expression levels of specific genes (RUNX2, CollagenI, and ALP are osteogenic genes, and SOX9, CollagenP, and aggrecan are chondrogenic genes) in the control group (mesenchymal stem cells were simply seeded in culture dishes) were measured.

图14为脱细胞骺软骨联合骨脱细胞材料体外成骨、成软骨诱导评估图；将小鼠骨间充质干细胞种植在脱细胞骺软骨联合骨脱细胞材料上3、7、14天，收集细胞并提取RNA后进行PCR试验后测定与空白组(间充质干细胞单纯种植于培养皿中)特定基因(RUNX2、CollagenI、ALP为成骨基因，SOX9、CollagenΠ、aggrecan为成软骨基因)的相对表达量。

The results showed that decellularized epiphyseal cartilage combined with decellularized bone material significantly promoted bone and cartilage formation.

结果显示脱细胞骺软骨联合骨脱细胞材料具有明显促进骨和软骨生成作用。

[n0073]

Figure 15 shows the in vivo osteogenic and chondrogenic induction tissue evaluation of decellularized epiphyseal cartilage combined with decellularized bone material. After 4 and 8 weeks of implantation of decellularized epiphyseal cartilage with bone into the distal femoral articular surface defect of adult New Zealand white rabbits, the experimental site was removed and subjected to HE staining and Safranin-Fix-Green staining to preliminarily determine the bone and cartilage repair effect.

图15为脱细胞骺软骨联合骨脱细胞材料在体成骨、成软骨诱导组织评估图；将脱细胞骺软骨连骨埋置于成年新西兰大白兔股骨远端关节面缺损中4周和8周后，将实验部位取出后，行HE染色及番红固绿染色以初步判断骨和软骨修复效果。

The results showed that at week 4, compared with the control group, the cartilage and subchondral bone began to repair initially; at week 8, the subchondral bone was basically repaired completely, and the cartilage was basically repaired except for the defect at the junction of the defect and the rest of the defect.

结果显示第4周时，相对空白组，可见软骨及软骨下骨开始初步修复；第8周时软骨下骨基本修复完全，软骨部分除缺损交界部位缺损虽未完全修复，但其余部位均基本修复完全。

In conclusion, decellularized epiphyseal cartilage combined with decellularized bone materials can promote the repair and regeneration of osteochondral defects.

综上所述，脱细胞骺软骨联合骨脱细胞材料能促进骨软骨缺损的修复再生。

[n0074]

Figures 16-18 show the quantitative evaluation of in vivo osteogenic and chondrogenic induction of decellularized epiphyseal cartilage combined with decellularized bone material. The area within 1 mm below the articular surface in Figure 15 is defined as the cartilage region, and the area 1-4 mm below the articular surface is defined as the subchondral bone region. The area occupied by cartilage in the cartilage region and the area occupied by cartilage and bone in the subchondral bone region are measured using quantitative methods to quantitatively evaluate the cartilage and bone repair effect at the defect site.

图16-18为脱细胞骺软骨联合骨脱细胞材料在体成骨、成软骨诱导定量评估图；将图15中的关节面以下1mm内作为软骨区域，关节面以下1-4mm作为软骨下骨区域，采用定量手法测定软骨区域内软骨所占面积、软骨下骨区域内软骨及骨所占面积，以定量评估缺损部位软骨及骨修复效果。

The results showed that at week 8, decellularized epiphyseal cartilage combined with decellularized bone material had a significant effect on promoting cartilage and bone formation.

结果显示在第8周时，脱细胞骺软骨联合骨脱细胞材料具有明显促软骨、促骨生成作用。

[n0075]

Example 2

实施例2

[n0076]

(1) Material collection: Take fresh femoral distal epiphyseal fusion bone from 3-month-old juvenile Large White pigs and rinse it 5 times with sterile PBS buffer at 4°C for 10 minutes each time to remove impurities such as hair, muscle, fascia, blood and tissue fluid from the surface; the size of the material is determined according to actual needs, usually a cylinder with a diameter of 5 mm and a height of 5 mm is taken.

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(1)取材：取新鲜3月龄幼年大白猪股骨远端骺软骨联合骨，在4℃低温环境下用无菌PBS缓冲液漂洗5次，每次10min，去除表面的毛发、肌肉、筋膜、血液和组织液等杂质；取材大小视实际需求确定，通常大小取直径为5mm，高度为5mm的圆柱体；

[n0077]

(2) Decellularization process:

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(2)脱细胞流程：

[n0078]

① Place the solution in 500ml of organic solvent solution (20% acetone solution) in a constant temperature shaker at 25°C and 60rpm for 2 hours to degrease, and then rinse with sterile PBS for 12 hours.

---

①、在500ml有机溶剂溶液(20%丙酮溶液)中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂2h，后用无菌PBS冲洗12h；

[n0079]

② After drying the material with sterile gauze, place it in liquid nitrogen for 30 minutes, then quickly remove it and place it in a 37°C water bath for 30 minutes; repeat 3 times; finally, rinse twice with sterile PBS for 5 minutes each time.

---

②、用无菌纱布将材料擦干后，置于液氮30min后，再迅速取出放置于37°C水浴30min；重复3次；最后用无菌PBS漂洗2次，每次5min；

[n0080]

③ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2% Triton-X100. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 48 h; then rinse with sterile PBS for 12 h.

---

③、在500ml含有体积浓度为2%Triton-X100的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡48h；再用无菌PBS冲洗12h；

[n0081]

④ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 5% SLES. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 36 h; then rinse with sterile PBS for 12 h.

---

④、在500ml含有质量浓度为5%SLES的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡36h；再用无菌PBS冲洗12h；

[n0082]

⑤ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HTHOPS). Place the solution in a constant temperature shaker at 25°C and 100 rpm for 6 h; then rinse with sterile PBS for 12 h.

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⑤、在500ml含有质量浓度为2%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HTHOPS)的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡6h；再用无菌PBS冲洗12h；

[n0083]

⑥ Add 10 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 100 ml of PBS buffer containing 0.01 mg/ml DNase I, and place it in a water bath at 37°C for 6 h.

---

⑥、在100ml含浓度为0.01mg/ml的DNase Ⅰ的PBS缓冲液中，加入10ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温37°C水浴箱中6h；

[n0084]

⑦ Place 500 ml of 50% ethanol in a constant temperature shaker at 25°C and 120 rpm for 24 hours.

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⑦、在500ml质量浓度为50%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡24h；

[n0085]

⑧. Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of sterile physiological saline. Place the solution in a constant temperature shaker at 37°C and 120 rpm for 12 h. Repeat this process 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

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⑧、在500ml无菌生理盐水中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于温度为37°C、转速为120rpm的恒温摇床中震荡12h，重复3次，即得到天然组织来源的骺软骨联合骨脱细胞材料。

[n0086]

Example 3

实施例3

[n0087]

(1) Material collection: Take fresh femoral distal epiphyseal fusion bone from 3-month-old juvenile Large White pigs and rinse it 5 times with sterile PBS buffer at 4°C for 10 minutes each time to remove impurities such as hair, muscle, fascia, blood and tissue fluid from the surface; the size of the material is determined according to actual needs, usually a cylinder with a diameter of 5 mm and a height of 5 mm is taken.

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(1)取材：取新鲜3月龄幼年大白猪股骨远端骺软骨联合骨，在4°C低温环境下用无菌PBS缓冲液漂洗5次，每次10min，去除表面的毛发、肌肉、筋膜、血液和组织液等杂质；取材大小视实际需求确定，通常大小取直径为5mm，高度为5mm的圆柱体；

[n0088]

(2) Decellularization process:

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(2)脱细胞流程：

[n0089]

① Place the solution in 500ml of organic solvent solution (30% acetone solution) in a constant temperature shaker at 25°C and 60rpm for 6 hours to degrease, and then rinse with sterile PBS for 12 hours.

---

①、在500ml有机溶剂溶液(30%丙酮溶液)中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂6h，后用无菌PBS冲洗12h；

[n0090]

② After drying the material with sterile gauze, place it in liquid nitrogen for 30 minutes, then quickly remove it and place it in a 37°C water bath for 30 minutes; repeat 3 times; finally, rinse twice with sterile PBS for 5 minutes each time.

---

②、用无菌纱布将材料擦干后，置于液氮30min后，再迅速取出放置于37°C水浴30min；重复3次；最后用无菌PBS漂洗2次，每次5min；

[n0091]

③ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% Triton-X100. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 24 h; then rinse with sterile PBS for 12 h.

---

③、在500ml含有体积浓度为1%Triton-X100的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡24h；再用无菌PBS冲洗12h；

[n0092]

④ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2% SLES. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 12 h; then rinse with sterile PBS for 12 h.

---

④、在500ml含有质量浓度为2%SLES的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡12h；再用无菌PBS冲洗12h；

[n0093]

⑤ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HTHOPS). Place the solution in a constant temperature shaker at 25°C and 100 rpm for 12 h; then rinse with sterile PBS for 12 h.

---

⑤、在500ml含有质量浓度为1%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HTHOPS)的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡12h；再用无菌PBS冲洗12h；

[n0094]

⑥ Add 10 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 100 ml of PBS buffer containing 0.01 mg/ml DNase I, and place it in a water bath at 37°C for 9 h.

---

⑥、在100ml含浓度为0.01mg/ml的DNase Ⅰ的PBS缓冲液中，加入10ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温37°C水浴箱中9h；

[n0095]

⑦ Place 500 ml of 50% ethanol in a constant temperature shaker at 25°C and 120 rpm for 48 hours.

---

⑦、在500ml质量浓度为50%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡48h；

[n0096]

⑧. Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of sterile physiological saline. Place the solution in a constant temperature shaker at 37°C and 120 rpm for 12 h. Repeat this process 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

---

⑧、在500ml无菌生理盐水中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于温度为37°C、转速为120rpm的恒温摇床中震荡12h，重复3次，即得到天然组织来源的骺软骨联合骨脱细胞材料。

[n0097]

Example 4

实施例4

[n0098]

(1) Material collection: Take fresh femoral distal epiphyseal fusion bone from 4-month-old young Large White pigs and rinse it 5 times with sterile PBS buffer at 4°C for 10 minutes each

time to remove impurities such as hair, muscle, fascia, blood and tissue fluid from the surface; the size of the material is determined according to actual needs, usually a cylinder with a diameter of 5mm and a height of 5mm is taken.

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(1)取材：取新鲜4月龄幼年大白猪股骨远端骺软骨联合骨，在4℃低温环境下用无菌PBS缓冲液漂洗5次，每次10min，去除表面的毛发、肌肉、筋膜、血液和组织液等杂质；取材大小视实际需求确定，通常大小取直径为5mm，高度为5mm的圆柱体；

[n0099]

(2) Decellularization process:

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(2)脱细胞流程：

[n0100]

① Place the solution in 500ml of organic solvent solution (40% acetone solution) in a constant temperature shaker at 25°C and 60rpm for 2 hours to degrease, and then rinse with sterile PBS for 12 hours.

---

①、在500ml有机溶剂溶液(40%丙酮溶液)中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂2h，后用无菌PBS冲洗12h；

[n0101]

② After drying the material with sterile gauze, place it in liquid nitrogen for 30 minutes, then quickly remove it and place it in a 37°C water bath for 30 minutes; repeat 3 times; finally, rinse twice with sterile PBS for 5 minutes each time.

---

②、用无菌纱布将材料擦干后，置于液氮30min后，再迅速取出放置于37°C水浴30min；重复3次；最后用无菌PBS漂洗2次，每次5min；

[n0102]

③ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2% Triton-X100. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 24 h; then rinse with sterile PBS for 12 h.

---

③、在500ml含有体积浓度为2%Triton-X100的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡24h；再用无菌PBS冲洗12h；

[n0103]

④ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 5% SLES. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 12 h; then rinse with sterile PBS for 12 h.

---

④、在500ml含有质量浓度为5%SLES的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡12h；再用无菌PBS冲洗12h；

[n0104]

⑤ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HTHOPS). Place the solution in a constant temperature shaker at 25°C and 100 rpm for 12 h; then rinse with sterile PBS for 12 h.

---

⑤、在500ml含有质量浓度为2%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HTHOPS)的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡12h；再用无菌PBS冲洗12h；

[n0105]

⑥ Add 10 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 100 ml of PBS buffer containing 0.01 mg/ml DNase I, and place it in a water bath at 37°C for 12 h.

---

⑥、在100ml含浓度为0.01mg/ml的DNase Ⅰ的PBS缓冲液中，加入10ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温37°C水浴箱中12h；

[n0106]

⑦ Place 500 ml of 70% ethanol in a constant temperature shaker at 25°C and 120 rpm for 48 hours.

---

⑦、在500ml质量浓度为70%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡48h；

[n0107]

⑧. Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of sterile physiological

saline. Place the solution in a constant temperature shaker at 37°C and 120 rpm for 12 h. Repeat this process 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

---

⑧、在500ml无菌生理盐水中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于温度为37°C、转速为120rpm的恒温摇床中震荡12h，重复3次，即得到天然组织来源的骺软骨联合骨脱细胞材料。

[n0108]

Example 5

实施例5

[n0109]

(1) Material collection: Take fresh femoral distal epiphyseal fusion bone from 4-month-old young Large White pigs and rinse it 5 times with sterile PBS buffer at 4°C for 10 minutes each time to remove impurities such as hair, muscle, fascia, blood and tissue fluid from the surface; the size of the material is determined according to actual needs, usually a cylinder with a diameter of 5mm and a height of 5mm is taken.

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(1)取材：取新鲜4月龄幼年大白猪股骨远端骺软骨联合骨，在4°C低温环境下用无菌PBS缓冲液漂洗5次，每次10min，去除表面的毛发、肌肉、筋膜、血液和组织液等杂质；取材大小视实际需求确定，通常大小取直径为5mm，高度为5mm的圆柱体；

[n0110]

(2) Decellularization process:

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(2)脱细胞流程：

[n0111]

① Place the solution in 500ml of organic solvent solution (40% acetone solution) in a constant temperature shaker at 25°C and 60rpm for 6 hours to degrease, and then rinse with sterile PBS for 12 hours.

---

①、在500ml有机溶剂溶液(40%丙酮溶液)中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂6h，后用无菌PBS冲洗12h；

[n0112]

② After drying the material with sterile gauze, place it in liquid nitrogen for 30 minutes, then quickly remove it and place it in a 37°C water bath for 30 minutes; repeat 3 times; finally, rinse twice with sterile PBS for 5 minutes each time.

---

②、用无菌纱布将材料擦干后，置于液氮30min后，再迅速取出放置于37°C水浴30min；重复3次；最后用无菌PBS漂洗2次，每次5min；

[n0113]

③ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% Triton-X100. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 72 h; then rinse with sterile PBS for 12 h.

---

③、在500ml含有体积浓度为1%Triton-X100的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡72h；再用无菌PBS冲洗12h；

[n0114]

④ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2%

SLES. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 48 h; then rinse with sterile PBS for 12 h.

---

④、在500ml含有质量浓度为2%SLES的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25℃、转速为100rpm的恒温摇床中震荡48h；再用无菌PBS冲洗12h；

[n0115]

⑤ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HTHOPS). Place the solution in a constant temperature shaker at 25°C and 100 rpm for 9 h; then rinse with sterile PBS for 12 h.

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⑤、在500ml含有质量浓度为1%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HTHOPS)的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25℃、转速为100rpm的恒温摇床中震荡9h；再用无菌PBS冲洗12h；

[n0116]

⑥ Add 10 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 100 ml of PBS buffer containing 0.01 mg/ml DNase I, and place it in a water bath at 37°C for 12 h.

---

⑥、在100ml含浓度为0.01mg/ml的DNase Ⅰ的PBS缓冲液中，加入10ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温37°C水浴箱中12h；

[n0117]

⑦ Place 500 ml of 60% ethanol in a constant temperature shaker at 25°C and 120 rpm for 24 hours.

---

⑦、在500ml质量浓度为60%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡24h；

[n0118]

⑧. Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of sterile physiological saline. Place the solution in a constant temperature shaker at 37°C and 120 rpm for 12 h. Repeat this process 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

---

⑧、在500ml无菌生理盐水中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于温度为37°C、转速为120rpm的恒温摇床中震荡12h，重复3次，即得到天然组织来源的骺软骨联合骨脱细胞材料。

[n0119]

Example 6

实施例6

[n0120]

(1) Material collection: Take fresh femoral distal epiphyseal fusion bone from 4-month-old young Large White pigs and rinse it 5 times with sterile PBS buffer at 4°C for 10 minutes each time to remove impurities such as hair, muscle, fascia, blood and tissue fluid from the surface; the size of the material is determined according to actual needs, usually a cylinder with a diameter of 5mm and a height of 5mm is taken.

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(1)取材：取新鲜4月龄幼年大白猪股骨远端骺软骨联合骨，在4°C低温环境下用无菌PBS缓冲液漂洗5次，每次10min，去除表面的毛发、肌肉、筋膜、血液和组织液等杂质；取材大小视实际需求确定，通常大小取直径为5mm，高度为5mm的圆柱体；

[n0121]

(2) Decellularization process:

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(2)脱细胞流程：

[n0122]

① Place the solution in 500 ml of organic solvent solution (50% ethanol solution) in a constant temperature shaker at 25°C and 60 rpm for 2 hours to degrease, and then rinse with sterile PBS for 12 hours.

---

①、在500ml有机溶剂溶液(50%乙醇溶液)中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂2h，后用无菌PBS冲洗12h；

[n0123]

② After drying the material with sterile gauze, place it in liquid nitrogen for 30 minutes, then quickly remove it and place it in a 37°C water bath for 30 minutes; repeat 3 times; finally, rinse twice with sterile PBS for 5 minutes each time.

---

②、用无菌纱布将材料擦干后，置于液氮30min后，再迅速取出放置于37°C水浴30min；重复3次；最后用无菌PBS漂洗2次，每次5min；

[n0124]

③ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2% Triton-X100. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 72 h; then rinse with sterile PBS for 12 h.

---

③、在500ml含有体积浓度为2%Triton-X100的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡72h；再用无菌PBS冲洗12h；

[n0125]

④ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 5% SLES. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 48 h; then rinse with sterile PBS for 12 h.

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④、在500ml含有质量浓度为5%SLES的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25℃、转速为100rpm的恒温摇床中震荡48h；再用无菌PBS冲洗12h；

[n0126]

⑤ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HTHOPS). Place the solution in a constant temperature shaker at 25°C and 100 rpm for 9 h; then rinse with sterile PBS for 12 h.

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⑤、在500ml含有质量浓度为2%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HTHOPS)的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25℃、转速为100rpm的恒温摇床中震荡9h；再用无菌PBS冲洗12h；

[n0127]

⑥ Add 10 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 100 ml of PBS buffer containing 0.01 mg/ml DNase I, and place it in a water bath at 37°C for 12 h.

---

⑥、在100ml含浓度为0.01mg/ml的DNase Ⅰ的PBS缓冲液中，加入10ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温37°C水浴箱中12h；

[n0128]

⑦ Place 500 ml of 60% ethanol in a constant temperature shaker at 25°C and 120 rpm for 48 hours.

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⑦、在500ml质量浓度为60%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡48h；

[n0129]

⑧. Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of sterile physiological saline. Place the solution in a constant temperature shaker at 37°C and 120 rpm for 12 h. Repeat this process 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

---

⑧、在500ml无菌生理盐水中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于温度为37°C、转速为120rpm的恒温摇床中震荡12h，重复3次，即得到天然组织来源的骺软骨联合骨脱细胞材料。

[n0130]

Example 7

实施例7

[n0131]

(1) Material collection: Take fresh femoral distal epiphyseal fusion bone from 4-month-old young Large White pigs and rinse it 5 times with sterile PBS buffer at 4°C for 10 minutes each time to remove impurities such as hair, muscle, fascia, blood and tissue fluid from the surface; the size of the material is determined according to actual needs, usually a cylinder with a diameter of 5mm and a height of 5mm is taken.

---

(1)取材：取新鲜4月龄幼年大白猪股骨远端骺软骨联合骨，在4°C低温环境下用无菌PBS缓冲液漂洗5次，每次10min，去除表面的毛发、肌肉、筋膜、血液和组织液等杂质；取材大小视实际需求确定，通常大小取直径为5mm，高度为5mm的圆柱体；

[n0132]

(2) Decellularization process:

---

(2)脱细胞流程：

[n0133]

① Place the solution in 500 ml of organic solvent solution (50% ethanol solution) in a constant temperature shaker at 25°C and 60 rpm for 6 hours to degrease, and then rinse with sterile PBS for 12 hours.

---

①、在500ml有机溶剂溶液(50%乙醇溶液)中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂6h，后用无菌PBS冲洗12h；

[n0134]

② After drying the material with sterile gauze, place it in liquid nitrogen for 30 minutes, then quickly remove it and place it in a 37°C water bath for 30 minutes; repeat 3 times; finally, rinse twice with sterile PBS for 5 minutes each time.

---

②、用无菌纱布将材料擦干后，置于液氮30min后，再迅速取出放置于37°C水浴30min；重复3次；最后用无菌PBS漂洗2次，每次5min；

[n0135]

③ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% Triton-X100. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 48 h; then rinse with sterile PBS for 12 h.

---

③、在500ml含有体积浓度为1%Triton-X100的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡48h；再用无菌PBS冲洗12h；

[n0136]

④ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 3% SLES. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 36 h; then rinse with sterile PBS for 12 h.

---

④、在500ml含有质量浓度为3%SLES的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡36h；再用无菌PBS冲洗12h；

[n0137]

⑤ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HTHOPS). Place the solution in a constant temperature shaker at 25°C and 100 rpm for 6 h; then rinse with sterile PBS for 12 h.

---

⑤、在500ml含有质量浓度为1%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HTHOPS)的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡6h；再用无菌PBS冲洗12h；

[n0138]

⑥ Add 10 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 100 ml of PBS buffer containing 0.01 mg/ml DNase I, and place it in a water bath at 37°C for 12 h.

---

⑥、在100ml含浓度为0.01mg/ml的DNase Ⅰ的PBS缓冲液中，加入10ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温37°C水浴箱中12h；

[n0139]

⑦ Place 500 ml of 50% ethanol in a constant temperature shaker at 25°C and 120 rpm for 36 hours.

---

⑦、在500ml质量浓度为50%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡36h；

[n0140]

⑧. Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of sterile physiological saline. Place the solution in a constant temperature shaker at 37°C and 120 rpm for 12 h. Repeat this process 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

---

⑧、在500ml无菌生理盐水中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于温度为37°C、转速为120rpm的恒温摇床中震荡12h，重复3次，即得到天然组织来源的骺软骨联合骨脱细胞材料。

[n0141]

Example 8

实施例8

[n0142]

(1) Material collection: Take fresh femoral distal epiphyseal fusion bone from 3-month-old juvenile Large White pigs and rinse it 5 times with sterile PBS buffer at 4°C for 10 minutes each time to remove impurities such as hair, muscle, fascia, blood and tissue fluid from the surface; the size of the material is determined according to actual needs, usually a cylinder with a diameter of 5 mm and a height of 5 mm is taken.

---

(1)取材：取新鲜3月龄幼年大白猪股骨远端骺软骨联合骨，在4°C低温环境下用无菌PBS缓冲液漂洗5次，每次10min，去除表面的毛发、肌肉、筋膜、血液和组织液等杂质；取材大小视实际需求确定，通常大小取直径为5mm，高度为5mm的圆柱体；

[n0143]

(2) Decellularization process:

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(2)脱细胞流程：

[n0144]

① Place the solution in 500 ml of organic solvent solution (70% ethanol solution) in a constant temperature shaker at 25°C and 60 rpm for 2 hours to degrease, and then rinse with sterile PBS for 12 hours.

---

①、在500ml有机溶剂溶液(70%乙醇溶液)中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂2h，后用无菌PBS冲洗12h；

[n0145]

② After drying the material with sterile gauze, place it in liquid nitrogen for 30 minutes, then quickly remove it and place it in a 37°C water bath for 30 minutes; repeat 3 times; finally, rinse twice with sterile PBS for 5 minutes each time.

---

②、用无菌纱布将材料擦干后，置于液氮30min后，再迅速取出放置于37°C水浴30min；重复3次；最后用无菌PBS漂洗2次，每次5min；

[n0146]

③ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% Triton-X100. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 48 h; then rinse with sterile PBS for 12 h.

---

③、在500ml含有体积浓度为1%Triton-X100的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡48h；再用无菌PBS冲洗12h；

[n0147]

④ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 4% SLES. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 36 h; then rinse with sterile PBS for 12 h.

---

④、在500ml含有质量浓度为4%SLES的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡36h；再用无菌PBS冲洗12h；

[n0148]

⑤ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HTHOPS). Place the solution in a constant temperature shaker at 25°C and 100 rpm for 6 h; then rinse with sterile PBS for 12 h.

---

⑤、在500ml含有质量浓度为1%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HTHOPS)的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡6h；再用无菌PBS冲洗12h；

[n0149]

⑥ Add 10 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 100 ml of PBS buffer containing 0.01 mg/ml DNase I, and place it in a water bath at 37°C for 12 h.

---

⑥、在100ml含浓度为0.01mg/ml的DNase Ⅰ的PBS缓冲液中，加入10ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温37°C水浴箱中12h；

[n0150]

⑦ Place 500 ml of 60% ethanol in a constant temperature shaker at 25°C and 120 rpm for 36 hours.

---

⑦、在500ml质量浓度为60%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡36h；

[n0151]

⑧. Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of sterile physiological saline. Place the solution in a constant temperature shaker at 37°C and 120 rpm for 12 h. Repeat this process 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

---

⑧、在500ml无菌生理盐水中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于温度为37°C、转速为120rpm的恒温摇床中震荡12h，重复3次，即得到天然组织来源的骺软骨联合骨脱细胞材料。

[n0152]

Example 9

实施例9

[n0153]

(1) Material collection: Take fresh femoral distal epiphyseal fusion bone from 3-month-old juvenile Large White pigs and rinse it 5 times with sterile PBS buffer at 4°C for 10 minutes each

time to remove impurities such as hair, muscle, fascia, blood and tissue fluid from the surface; the size of the material is determined according to actual needs, usually a cylinder with a diameter of 5mm and a height of 5mm is taken.

---

(1)取材：取新鲜3月龄幼年大白猪股骨远端骺软骨联合骨，在4℃低温环境下用无菌PBS缓冲液漂洗5次，每次10min，去除表面的毛发、肌肉、筋膜、血液和组织液等杂质；取材大小视实际需求确定，通常大小取直径为5mm，高度为5mm的圆柱体；

[n0154]

(2) Decellularization process:

---

(2)脱细胞流程：

[n0155]

① Place the solution in 500 ml of organic solvent solution (70% ethanol solution) in a constant temperature shaker at 25°C and 60 rpm for 6 hours to degrease, and then rinse with sterile PBS for 12 hours.

---

①、在500ml有机溶剂溶液(70%乙醇溶液)中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂6h，后用无菌PBS冲洗12h；

[n0156]

② After drying the material with sterile gauze, place it in liquid nitrogen for 30 minutes, then quickly remove it and place it in a 37°C water bath for 30 minutes; repeat 3 times; finally, rinse twice with sterile PBS for 5 minutes each time.

---

②、用无菌纱布将材料擦干后，置于液氮30min后，再迅速取出放置于37°C水浴30min；重复3次；最后用无菌PBS漂洗2次，每次5min；

[n0157]

③ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% Triton-X100. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 48 h; then rinse with sterile PBS for 12 h.

---

③、在500ml含有体积浓度为1%Triton-X100的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡48h；再用无菌PBS冲洗12h；

[n0158]

④ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2% SLES. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 36 h; then rinse with sterile PBS for 12 h.

---

④、在500ml含有质量浓度为2%SLES的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡36h；再用无菌PBS冲洗12h；

[n0159]

⑤ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HTHOPS). Place the solution in a constant temperature shaker at 25°C and 100 rpm for 6 h; then rinse with sterile PBS for 12 h.

---

⑤、在500ml含有质量浓度为1%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HTHOPS)的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡6h；再用无菌PBS冲洗12h；

[n0160]

⑥ Add 10 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 100 ml of PBS buffer containing 0.01 mg/ml DNase I, and place it in a water bath at 37°C for 12 h.

---

⑥、在100ml含浓度为0.01mg/ml的DNase Ⅰ的PBS缓冲液中，加入10ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温37°C水浴箱中12h；

[n0161]

⑦ Place 500 ml of 70% ethanol in a constant temperature shaker at 25°C and 120 rpm for 36 hours.

---

⑦、在500ml质量浓度为70%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡36h；

[n0162]

⑧. Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of sterile physiological

saline. Place the solution in a constant temperature shaker at 37°C and 120 rpm for 12 h. Repeat this process 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

---

⑧、在500ml无菌生理盐水中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于温度为37°C、转速为120rpm的恒温摇床中震荡12h，重复3次，即得到天然组织来源的骺软骨联合骨脱细胞材料。

[n0163]

Example 10

实施例10

[n0164]

(1) Material collection: Take fresh femoral distal epiphyseal fusion bone from 3-month-old juvenile Large White pigs and rinse it 5 times with sterile PBS buffer at 4°C for 10 minutes each time to remove impurities such as hair, muscle, fascia, blood and tissue fluid from the surface; the size of the material is determined according to actual needs, usually a cylinder with a diameter of 5 mm and a height of 5 mm is taken.

---

(1)取材：取新鲜3月龄幼年大白猪股骨远端骺软骨联合骨，在4°C低温环境下用无菌PBS缓冲液漂洗5次，每次10min，去除表面的毛发、肌肉、筋膜、血液和组织液等杂质；取材大小视实际需求确定，通常大小取直径为5mm，高度为5mm的圆柱体；

[n0165]

(2) Decellularization process:

---

(2)脱细胞流程：

[n0166]

① Place the solution in 500 ml of organic solvent solution (60% ethanol solution) in a constant temperature shaker at 25°C and 60 rpm for 4 hours to degrease, and then rinse with sterile PBS for 12 hours.

---

①、在500ml有机溶剂溶液(60%乙醇溶液)中，置于恒温25°C、转速为60rpm的恒温摇床中震荡脱脂4h，后用无菌PBS冲洗12h；

[n0167]

② After drying the material with sterile gauze, place it in liquid nitrogen for 30 minutes, then quickly remove it and place it in a 37°C water bath for 30 minutes; repeat 3 times; finally, rinse twice with sterile PBS for 5 minutes each time.

---

②、用无菌纱布将材料擦干后，置于液氮30min后，再迅速取出放置于37°C水浴30min；重复3次；最后用无菌PBS漂洗2次，每次5min；

[n0168]

③ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% Triton-X100. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 48 h; then rinse with sterile PBS for 12 h.

---

③、在500ml含有体积浓度为1%Triton-X100的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡48h；再用无菌PBS冲洗12h；

[n0169]

④ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 2%

SLES. Place the solution in a constant temperature shaker at 25°C and 100 rpm for 36 h; then rinse with sterile PBS for 12 h.

---

④、在500ml含有质量浓度为2%SLES的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25℃、转速为100rpm的恒温摇床中震荡36h；再用无菌PBS冲洗12h；

[n0170]

⑤ Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of PBS buffer containing 1% sodium 3-allyloxy-2-hydroxy-1-propanesulfonate (HTHOPS). Place the solution in a constant temperature shaker at 25°C and 100 rpm for 6 h; then rinse with sterile PBS for 12 h.

---

⑤、在500ml含有质量浓度为1%的3-烯丙氧基-2-羟基-1-丙烷磺酸钠盐(HTHOPS)的PBS缓冲液中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温25°C、转速为100rpm的恒温摇床中震荡6h；再用无菌PBS冲洗12h；

[n0171]

⑥ Add 10 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 100 ml of PBS buffer containing 0.01 mg/ml DNase I, and place it in a water bath at 37°C for 12 h.

---

⑥、在100ml含浓度为0.01mg/ml的DNase Ⅰ的PBS缓冲液中，加入10ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于恒温37°C水浴箱中12h；

[n0172]

⑦ Place 500 ml of 70% ethanol in a constant temperature shaker at 25°C and 120 rpm for 36 hours.

---

⑦、在500ml质量浓度为70%的乙醇中，置于恒温25°C、转速为120rpm的恒温摇床中震荡36h；

[n0173]

⑧. Add 50 ml of a mixed antibacterial solution of penicillin and streptomycin with concentrations of 100 U/ml and 100 μg/ml, respectively, to 500 ml of sterile physiological saline. Place the solution in a constant temperature shaker at 37°C and 120 rpm for 12 h. Repeat this process 3 times to obtain epiphyseal cartilage combined with decellularized bone material derived from natural tissue.

---

⑧、在500ml无菌生理盐水中，加入50ml浓度分别为100U/ml，100μg/ml的青霉素和链霉素的混合抗菌溶液，置于温度为37℃、转速为120rpm的恒温摇床中震荡12h，重复3次，即得到天然组织来源的骺软骨联合骨脱细胞材料。

[n0174]

Examples 2-10 all successfully prepared epiphyseal cartilage combined with bone decellularization material with complete decellularization, well-preserved three-dimensional scaffold structure and active ingredients, and certain biological activities (such as osteogenic and chondrogenic activities), and the corresponding results were basically consistent with those of Example 1.

实施例2-10均能制备出脱细胞完全、支架三维结构及活性成分保留完好、具有一定生物活性(如成骨及成软骨活性)的骺软骨联合骨脱细胞材料，相应结果与实施例1基本一致。

[n0175]

The above description of the disclosed embodiments enables those skilled in the art to implement or use the present invention.

对所公开的实施例的上述说明，使本领域专业技术人员能够实现或使用本发明。

Various modifications to these embodiments will be readily apparent to those skilled in the art, and the general principles defined herein may be implemented in other embodiments without departing from the spirit or scope of the invention.

对这些实施例的多种修改对本领域的专业技术人员来说将是显而易见的，本文中所定义的一般原理可以在不脱离本发明的精神或范围的情况下，在其它实施例中实现。

Therefore, the present invention is not to be limited to the embodiments shown herein, but is to be accorded the widest scope consistent with the principles and novel features disclosed herein.

因此，本发明将不会被限制于本文所示的这些实施例，而是要符合与本文所公开的原理和新颖特点相一致的最宽的范围。