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DESCRIPTION CN104307045A

A naturally derived decellularized periosteum material and its preparation method

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一种天然组织来源的脱细胞骨膜材料及其制备方法

[0001]

Technical Field

技术领域

[0002]

This invention relates primarily to the biological field for tissue or organ repair and regeneration, specifically to a decellularized periosteum material derived from natural tissue and its preparation method.

本发明主要涉及用于组织或器官修复及其再生的生物领域，具体是一种天然组织来源的脱细胞骨膜材料极其制备方法。

[0003]

Background Technology

背景技术

[0004]

Nonunion, delayed union, and bone defects are common and complex orthopedic conditions that not only perplex many surgeons but also pose significant health threats and heavy financial burdens to patients.

骨不愈合、延迟愈合和骨缺损等是临床上常见的骨科疑难疾病，这不仅困绕的许多外科医生，更给患者带来巨大健康威胁和沉重的经济负担。

Currently, traditional treatment methods for the above-mentioned diseases, such as reconstructive surgery, autologous bone transplantation, and joint replacement, all have many problems.

而目作为前临床上针对上述疾病的治疗手段，再次修复手术、自体骨移植、关节置换等传统治疗方法均存在着诸多问题。

For example, autologous bone transplantation has a series of problems such as difficulty in obtaining materials and large surgical trauma.

例如，自体骨移植存在取材困难，手术创伤大等一系列问题。

[0005]

In addition to being rich in blood vessels and nerves, providing nutrition and sensation, the periosteum also contains osteoblasts, which participate in bone thickening and growth, playing an important role in bone growth (lengthening and thickening) and proliferation (fracture healing).

骨膜除了富有血管、神经，有营养和感觉作用，更具有造骨细胞的功能，参与骨的增粗生长，对骨的生长（长长，长粗）和增生（断裂愈合）有重要作用。

In recent years, an increasing number of studies have used periosteal transplantation to treat bone defects and nonunion, achieving good results.

近年来越来越多的研究将骨膜移植用于治疗骨缺损和骨不愈合取得了较好的成果。

However, periosteal transplantation faces challenges such as insufficient donors, damage to the donor site's structure and function, and immune rejection, making it difficult to carry out on a large scale.

然而骨膜移植存在供体不足、供区结构功能损害及免疫排斥反应等问题使其难以广泛开展。

[0006]

The emergence of regenerative medicine and tissue engineering technologies has brought new ideas to the treatment of diseases such as nonunion, delayed healing, and bone defects.

再生医学和组织工程技术的出现使为治愈骨不愈合、延迟愈合和骨缺损等疾病带来了全新的思路。

In the field of bone tissue engineering, artificial biomimetic periosteum that simulates the microstructure of the periosteum is attracting increasing attention. For example, Ryu YM et al.

used a three-dimensional collagen scaffold to induce periosteal-derived cells to differentiate into osteoblasts. In addition, Zhao L et al. also used tissue engineering techniques to culture degenerative stem cells from the submucosa of the small intestine in an attempt to construct an artificial periosteum to repair bone defects. However, due to the complex three-dimensional structure of the natural periosteum and many of its components that are not yet fully understood, the results are often unsatisfactory. For example, the application of artificial materials is greatly limited due to problems such as biomechanics, biocompatibility, and material properties. Therefore, finding materials that truly conform to the normal biological and mechanical properties of the periosteum to cure diseases such as nonunion, delayed healing, and bone defects is a medical problem that urgently needs to be solved.

骨组织工程领域中，模拟骨膜微结构的人工仿生骨膜越来越受到人们的关注。 例如，Ryu YM等人利用三维胶原支架诱导骨膜来源的细胞向成骨分化。 此外，Zhao L等人也利用组织工程技术采用小肠粘膜下层组织来培养简从质干细胞尝试构建人工骨膜从而修复骨缺损。 但是由于天然骨膜具有复杂的三维结构和许多目前尚未明确的具体组成成分使得效果总是不尽如人意。 例如，人工材料由于生物力学、生物相容性、材料性质等问题使其运用受到很大限制。 因此找到真正符合正常骨膜生物学和力学性质的材料，从而治愈骨不愈合、延迟愈合和骨缺损等疾病是一个亟待解决医学难题。

[0007]

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

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。

ECM can regulate a range of factors required for tissue or organ development and repair, including biophysical stimulation, biochemical and molecular signals, thereby achieving tissue or organ recovery and regeneration. Therefore, ECM, as a novel natural biomaterial, is being widely used in the repair and regeneration of a range of tissues and organs, such as heart valves, trachea, muscles, tendons, and cartilage. Cell-decellularized dermal matrix has even been used clinically (trade name AlloDerm). There are currently no reports on the preparation of naturally derived decellularized periosteal materials.

ECM可调节生物物理刺激、生物化学及分子信号等一系列组织或器官发育及修复所需的各种因素，从而实现组织或器官的恢复和再生。 因此ECM作为一个新型的天然生物材料正被广泛的运用于心脏瓣膜、气管、肌肉、肌腱、软骨等一系列组织或器官的修复及再生。 去细胞的真皮基质甚至已用于临床(商品名AlloDerm)。 而目前尚无关于天然来源的骨膜脱细胞材料制备的报道。

[0008]

Summary of the Invention

发明内容

[0009]

This invention addresses the shortcomings of existing technologies by providing a method for preparing a decellularized periosteal material that is suitable for the growth of periosteal cells and various types of stem cells, has osteogenic induction effects, and can treat diseases such as nonunion, delayed healing, and bone defects.

本发明针对现有技术的不足，提供一种可适宜骨膜细胞和各类型干细胞生长、具有成骨诱导作用、可治疗骨不愈合、延迟愈合和骨缺损等疾病的骨膜脱细胞材料的制备方法。

[0010]

A decellularized periosteum material derived from natural tissue and its preparation method, specifically including the following steps:

一种天然组织来源的脱细胞骨膜材料及其制备方法，具体包括以下步骤：

[0011]

(1) Take the periosteum of any mammalian limb bone, remove bone fragments, rinse three times with sterile PBS for 20 minutes each time to remove blood, residual muscle tissue and fat tissue; then rinse with deionized water for 4 hours.

（1）取任一哺乳动物四肢骨骨膜，摘除骨性碎片，无菌PBS漂洗3次，每次漂洗20分钟，去除血液、残余肌肉组织和脂肪组织；再用去离子水冲洗4小时；

[0012]

(2) In a 5% PBS buffer containing 10 KIU/ml protease inhibitor, shake at 45°C for 1 hour at 200 rpm on a shaker; then rinse with deionized water for 4 hours.

（2）在浓度为5%的含10KIU/ml 蛋白酶抑制剂的PBS缓冲液，恒温45 ^o C摇床200rpm震荡1小时；用去离子水冲洗4小时；

[0013]

(3) Add 10 KIU/ml and 10 g/ml mixed antibacterial solution to 5% Triton X-100 PBS buffer, with a volume ratio of buffer to mixed antibacterial solution of 5:1, and shake at 250 rpm for 48 hours at a constant temperature of 45°C; then rinse with deionized water for 4 hours. The mixed antibacterial solution is composed of penicillin and streptomycin, with a volume ratio of penicillin to streptomycin of 1:1.

（3）在浓度为5%的含Triton X-100的PBS缓冲液，加入10KIU/ml，10g/ml的混合抗菌液，缓冲液和混合抗菌液体积比为5：1，恒温45 ^o C摇床250rpm震荡48小时；再用去离子水冲洗4小时，所述的混合抗菌液由青霉素和链霉素组成，青霉素和链霉素的体积比为1：1；

[0014]

(4) Add 10 KIU/ml and 10 g/ml mixed antibacterial solution to 10% SDS-containing PBS buffer, with a volume ratio of buffer to mixed antibacterial solution of 5:1, and shake at 250 rpm for 48 hours at a constant temperature of 45°C; rinse with deionized water for 4 hours. The mixed antibacterial solution is composed of penicillin and streptomycin, with a volume ratio of penicillin to streptomycin of 1:1.

（4）在浓度为10%的含SDS的PBS缓冲液，加入10KIU/ml，10g/ml的混合抗菌液，缓冲液和混合抗菌液体积比为5：1，恒温45 ^o C摇床250rpm震荡48小时；用去离子水冲洗4小时，所述的混合抗菌液由青霉素和链霉素组成，青霉素和链霉素的体积比为1：1；

[0015]

(5) Add 10 KIU/ml and 10 g/ml mixed antibacterial solution to PBS buffer containing DNase at a concentration of 1.5 mg/ml. The volume ratio of buffer to mixed antibacterial solution is 5:1. Shake at 250 rpm for 12 hours at 37°C. After rinsing with deionized water for 4 hours, a whole

intervertebral disc decellularized scaffold is obtained. The mixed antibacterial solution is composed of penicillin and streptomycin, and the volume ratio of penicillin to streptomycin is 1:1.

（5）在浓度为1. 5mg/ml的含DNA酶的PBS缓冲液，加入10KIU/ml，10g/ml的混合抗菌液，缓冲液和混合抗菌液体积比为5：1，37 ^o C摇床250rpm震荡12小时；用去离子水冲洗4小时后得到全椎间盘脱细胞支架，所述的混合抗菌液由青霉素和链霉素组成，青霉素和链霉素的体积比为1：1。

[0016]

The main advantages of this invention are as follows:

本发明的主要优点如下：

[0017]

(1) Decellularization technology can preserve the integrity of the original ECM while removing xenogeneic cells. It has a good extracellular microenvironment, biochemical factors and biomechanical properties, and can simulate the normal periosteum composition and structure to the greatest extent.

（1）脱细胞技术在去除异种细胞的同时，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膜成分和结构；

[0018]

(2) The material of the present invention can not only be used to implant various stem cells (embryonic stem cells, bone marrow mesenchymal stem cells (MSCs), adipose mesenchymal stem cells, etc.) and intervertebral disc cells to achieve normal intervertebral disc reconstruction and to customize a whole intervertebral disc that is individualized and transplantable for patients, but can also be ground into powder to dissolve the biochemical factors contained in normal intervertebral discs for the treatment of degenerated intervertebral discs.

（2）本发明材料不仅可以用于种植各种干细胞（胚胎干细胞、骨髓间充质干细胞（MSCs）、脂肪间充质干细胞等）和椎间盘细胞以实现正常椎间盘重构，为病人订制具有个体化、可供移植的全椎间盘，还可以将其研磨制成粉末，将正常椎间盘所含有的生化因子溶解用于治疗退变的椎间盘；

[0019]

(3) The periosteum used in this invention is a naturally sourced biological material with good biocompatibility and wide availability. The raw materials are widely available and can be mass-produced.

（3）本发明所采用的骨膜是天然来源的生物材料，具有很好的生物相容性和取材广泛性，且原材料来源广泛，可批量生产；

[0020]

(4) Periosteum materials obtained by decellularization technology do not contain antigens such as cells, which can minimize the immune rejection reaction of the recipient. At the same time, they do not contain harmful components such as bacteria and viruses, and have high biological safety.

（4）采用脱细胞技术获得的骨膜材料不含细胞等抗原物质，可使受体的免疫排斥反应降到最低限度，同时可不含细菌病毒等有害成分生物安全性高。

[0021]

Attached Figure Description

附图说明

[0022]

Figure 1 is a general appearance diagram of the decellularized periosteum material of the present invention;

图1是本发明的骨膜脱细胞材料大体外观图；

[0023]

Figure 2 shows the HE staining of the cross section of the decellularized periosteum material, which showed no cells and no residual cell nuclear components.

图2是骨膜脱细胞材料横切面的HE染色无细胞及无细胞核成分残留图；

[0024]

Figure 3 shows the HE staining of the longitudinal section of the decellularized periosteum material, which showed no cells and no residual cell nuclear components.

图3是骨膜脱细胞材料纵切面的HE染色无细胞及无细胞核成分残留图；

[0025]

Figure 4 shows the Alcian blue staining of a cross-section of the decellularized periosteum material, which retains a large amount of glycosaminoglycans.

图4是骨膜脱细胞材料横切面的阿尔新蓝染色保留大量糖胺聚糖成分图；

[0026]

Figure 5 shows the Alcian blue staining of a longitudinal section of the decellularized periosteum material, which retains a large amount of glycosaminoglycans.

图5是骨膜脱细胞材料纵切面的阿尔新蓝染色保留大量糖胺聚糖成分图；

[0027]

Figure 6 is a quantitative detection chart of collagen content in decellularized periosteum material;

图6是是骨膜脱细胞材料胶原蛋白含量定量检测图；

[0028]

Figure 7 shows that the DNA quantitative detection of decellularized periosteum material contained almost no DNA component;

图7是骨膜脱细胞材料DNA定量检测几乎不含有DNA成分图；

[0029]

Figure 8 is a cross-sectional view of the decellularized periosteum material, showing the complete preservation of collagen fiber arrangement and three-dimensional spatial structure as detected by scanning electron microscopy.

图8是骨膜脱细胞材料横切面扫描电镜检测胶原纤维排布和空间立体结构完整保留图；

[0030]

Figure 9 is a scanning electron microscope image of the longitudinal section of the decellularized periosteum material, showing the complete preservation of collagen fiber arrangement and three-dimensional spatial structure.

图9是骨膜脱细胞材料纵切面扫描电镜检测胶原纤维排布和空间立体结构完整保留图；

[0031]

Figure 10 shows the proliferation of bone marrow mesenchymal stem cells under different scaffold extract concentrations as detected by CCK-8 assay.

图10是CCK-8检测不同支架浸提液浓度下骨髓间充质干细胞的增值情况图；

[0032]

Figure 11. Growth of Live/Dead cell-stained bone marrow mesenchymal stem cells on a decellularized periosteum scaffold.

图11 Live/Dead细胞染色骨髓间充质干细胞在骨膜脱细胞材料支架上的生长情况图。

[0033]

The present invention will now be described in detail with reference to the accompanying drawings and embodiments, but the implementation of the present invention is not limited thereto.

下面结合附图及实施例对本发明进行详细描述，但本发明的实施不仅限于此。

[0034]

Specific Implementation Plan 1

具体实施方案一

[0035]

1. Preparation of decellularized periosteum matrix

1、制备骨膜脱细胞基质

[0036]

(1) Material collection: Take the periosteum of the long bones of the limbs of healthy domestic pigs (male or female), remove bone fragments, rinse thoroughly with sterile PBS to remove blood and other impurities. The periosteum is about 6cm long, 3cm wide and 5mm thick.

（1）取材：取健康家猪（雌雄不限）四肢长骨的骨膜，摘除骨性碎片，无菌PBS充分漂洗去除血液和其他杂质，骨膜长约6cm，宽约3cm，厚度约5mm。

[0037]

(2) The decellularization steps are as follows:

（2）脱细胞步骤如下：

[0038]

Step 1: Place 200 ml of 5% PBS buffer containing 10 KIU/ml protease inhibitor at 45°C and shake at 250 rpm for 4 hours; then rinse with deionized water for 4 hours.

步骤一:在200ml浓度为5%的含10KIU/ml 蛋白酶抑制剂的PBS缓冲液，恒温45 ^o C摇床250rpm震荡4小时；再用去离子水冲洗4小时；

[0039]

Step 2: Add 100 ml of penicillin and streptomycin (10 KIU/ml, 10 g/ml) mixed antibacterial solution to 500 ml of 5% Triton X-100 PBS buffer and incubate at 45°C with shaking at 250 rpm for 48 hours; rinse with deionized water for 4 hours.

步骤二：在500ml浓度为5%的含Triton X-100的PBS缓冲液，加入100ml青霉素和链霉素（10KIU/ml，10g/ml）混合抗菌液，恒温45 ^o C摇床250rpm震荡48小时；用去离子水冲洗4小时；

[0040]

Step 3: Add 200 ml of penicillin and streptomycin (10 KIU/ml, 10 g/ml) mixed antibacterial solution to 1000 ml of 10% SDS-containing PBS buffer, and incubate at 45°C with shaking at 250 rpm for 48 hours; rinse with deionized water for 4 hours.

步骤三：在1000ml浓度为10%的含SDS的PBS缓冲液，加入200ml青霉素和链霉素（10KIU/ml，10g/ml）混合抗菌液，恒温45 ^o C摇床250rpm震荡48小时；用去离子水冲洗4小时；

[0041]

Step 4: Add 50 ml of penicillin and streptomycin (10 KIU/ml, 10 g/ml) mixed antibacterial solution to 250 ml of PBS buffer containing DNase at a concentration of 1.5 mg/ml. Shake at 250 rpm for 12 hours at 37°C. Rinse with deionized water for 4 hours to obtain a whole intervertebral disc decellularized scaffold, as shown in Figure 1.

步骤四：用250ml浓度为1. 5mg/ml的含DNA酶的PBS缓冲液，加入50ml青霉素和链霉素（10KIU/ml，10g/ml）混合抗菌液，37 ^o C摇床250rpm震荡12小时后，用去离子水冲洗4小时得到全椎间盘脱细胞支架，如图1所示；

[0042]

(3) Histological evaluation of the decellularized periosteal scaffold

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（3）骨膜脱细胞支架的组织学评价

[0043]

Figures 2 and 3 are 100x magnification images of the decellularized periosteal scaffold (cross section and cross section) of the present invention stained with HE, showing no residual cells and no cell nuclei, while retaining a large amount of collagen fiber components; Figures 4 and 5 are 100x magnification images of the decellularized periosteal scaffold (cross section and cross section) of the present invention stained with Alcian blue, showing a large amount of glycosaminoglycan components.

图2和图3是本发明骨膜脱细胞支架（横切面和从切面）HE染色放大100倍无细胞及无细胞核成分残留图，保留大量胶原纤维成分；图4和图5是本发明骨膜脱细胞支架（横切面和从切面）阿尔新蓝染色放大100倍保留大量糖胺聚糖成分图。

Figure 6 is a quantitative detection diagram of collagen content in the decellularized periosteum material of the present invention. The collagen content of the material is not significantly reduced compared with normal tissue.

图6是本发明骨膜脱细胞材料胶原蛋白含量定量检测图，材料胶原蛋白含量与正常组织比较无明显减少。

[0044]

(4) Quantitative detection of antigen components in decellularized periosteal scaffolds

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（4）骨膜脱细胞支架的抗原成分定量检测

[0045]

Figure 7 shows the DNA quantification detection of the decellularized periosteum scaffold of the present invention, which contains almost no DNA components.

图7是本发明骨膜脱细胞支架DNA定量检测几乎不含有DNA成分图。

[0046]

(5) Observation of the ultrastructure of the decellularized periosteal scaffold

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（5）骨膜脱细胞支架的超微立体结构观察

[0047]

Figures 8 and 9 are scanning electron microscope images (cross-section and cross-section) of the decellularized intervertebral disc scaffold of the present invention, showing the complete preservation of collagen fiber arrangement and three-dimensional spatial structure.

图8和图9是本发明全椎间盘脱细胞支架（横切面和从切面）扫描电镜检测胶原纤维排布和空间立体结构完整保留图。

[0048]

(6) Biocompatibility evaluation of decellularized periosteal scaffold

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（6）骨膜脱细胞支架的生物相容性评价

[0049]

Figure 10 shows the proliferation of bone marrow mesenchymal stem cells under different scaffold extract concentrations using the CCK-8 periosteum decellularized scaffold of the present invention.

图10是本发明骨膜脱细胞支架CCK-8检测不同支架浸提液浓度下骨髓间充质干细胞的增值情况图。

Figure 11 shows the growth of bone marrow mesenchymal stem cells stained with Live/Dead cells on the decellularized periosteum scaffold of the present invention at 100x magnification on the whole intervertebral disc decellularized scaffold.

图11是本发明骨膜脱细胞支架Live/Dead细胞染色骨髓间充质干细胞在全椎间盘脱细胞支架上100倍的生长情况图。