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DESCRIPTION CN104307044A

A naturally derived decellularized intervertebral disc material and its preparation method

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一种天然组织来源的全椎间盘脱细胞材料及其制备方法

[0001]

Technical Field

技术领域

[0002]

This invention mainly relates to the biological field for tissue or organ repair and regeneration, specifically a decellularized whole intervertebral disc 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全椎间盘脱细胞材料极其制备方法。

[0003]

Background Technology

背景技术

[0004]

Intervertebral disc degeneration often causes back pain and disc herniation.

椎间盘退变常常引起背部疼痛及椎间盘突出。

According to statistics, about 84% of people in the United States have a history of lower back pain, and the related costs amount to as much as $100 billion annually.

据统计，在美国约有84%的人有过腰背部疼痛病史，与之相关花费每年更是高达1000亿美元。

Furthermore, intervertebral disc degeneration is considered an irreversible disease process that worsens with age.

并且椎间盘退变则被认为是一个不可逆的并且随年龄加重的病变过程。

Currently, traditional conservative treatments and surgical resection-fusion cannot achieve ideal radical cure results.

而目前传统的保守治疗和手术切除-融合均不能达到理想的根治效果。

[0005]

The emergence of regenerative medicine and tissue engineering technologies has made it possible to cure intervertebral disc degeneration.

再生医学和组织工程技术的出现使根治椎间盘退变成为可能。

In recent years, many researchers have attempted to treat intervertebral disc degeneration using techniques such as tissue engineering (metal-plastic composite intervertebral discs), molecular biology (injection of growth factors or cytokine inhibitors), and stem cell therapy.

However, due to the complex three-dimensional structure of the natural intervertebral disc (nucleus pulposus and annulus fibrosus) and many components that are not yet clearly defined, the results are always less than satisfactory. For example, the application of synthetic materials is greatly limited by problems such as biomechanics, biocompatibility, material properties, and long-term wear; while the short effective duration of growth factors and cytokine inhibitors also leads to poor treatment results; similarly, stem cell technology is currently unable to solve the series of disease processes caused by the destruction of intervertebral disc structure. Therefore, finding materials or treatment techniques that truly match the biological and mechanical properties of normal intervertebral discs to replace and reverse degenerated discs is a pressing medical challenge.

近年来，许多研究者尝试运用组织工程（金属-塑料复合椎间盘）、分子生物学（注射生长因子或细胞因子抑制剂）和干细胞治疗等技术治疗椎间盘退变。 但是由于天然椎间盘（髓核和纤维环）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，人工合成材料由于生物力学、生物相容性、材料性质和远期磨碎等问题使其运用受到很大限制；而生长因子和细胞因子抑制剂的有效作用时间短暂也使治疗效果不佳；同样干细胞技术目前还无法解决椎间盘结构破坏所导致的一系列疾病进程。 因此找到真正符合正常椎间盘生物学和力学性质的材料或是治疗技术，从而替换并逆转退变椎间盘是一个亟待解决医学难题。

[0006]

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. Currently, there are no reports on the preparation of decellularized materials from naturally derived complete intervertebral discs (nucleus pulposus and annulus fibrosus).

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。 而目前尚无关于天然来源的完整椎间盘（髓核和纤维环）脱细胞材料制备的报道。

[0007]

Summary of the Invention

发明内容

[0008]

The purpose of this invention is to address the shortcomings of existing technologies in terms of material composition, structure, and biomechanics of human intervertebral discs, as well as the defects of having only a single nucleus pulposus or fibrous scaffold. This invention provides a method for preparing a whole-disc decellularized material suitable for intervertebral disc cell growth, remodeling, and treatment of irreversible intervertebral disc degeneration.

本发明目的在于填补现有技术中，组织工作人椎间盘在材料组成、结构、生物力学方面的不足以及仅有单一髓核或纤维支架的缺陷，提供一种可适宜椎间盘细胞生长、重构及治疗不可逆性椎间盘退变的全椎间盘脱细胞材料的制备方法

[0009]

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

一种天然组织来源的全椎间盘脱细胞材料及其制备方法，具体包括以下步骤：

[0010]

(1) Take the intervertebral disc of the vertebrate, remove the bony fragments, rinse with sterile PBS 3 times, each time for 20 minutes, to remove blood, residual muscle tissue and ligaments, etc.

（1）取脊椎动物椎间盘，摘除骨性碎片，无菌PBS漂洗3次，每次漂洗20分钟，去除血液、残余肌肉组织和韧带等；

[0011]

(2) In a 10% PBS buffer containing 10 KIU/ml protease inhibitor, shake at 45°C for 4 hours on a shaker at 150 rpm; and rinse with PBS for 1 hour.

（2）在浓度为10%的含10KIU/ml 蛋白酶抑制剂的PBS缓冲液中，恒温 45 ^o C摇床150rpm震荡4小时；并用PBS冲洗1小时；

[0012]

(3) Add 10 KIU/ml and 10 g/ml mixed antibacterial solution to 4% Triton X-100 PBS buffer, with a volume ratio of buffer to mixed antibacterial solution of 5:1 and a ratio of buffer to mixed

antibacterial solution of 5:1. Shake at 150 rpm for 48 hours at a constant temperature of 45°C. Rinse with PBS for 1 hour. 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4%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，缓冲液和混合抗菌液比例5：1，恒温45 ^o C摇床150rpm震荡48小时；并用PBS冲洗1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；

[0013]

(4) Add 10 KIU/ml and 10 g/ml mixed antibacterial solution to 5% SDS-containing PBS buffer, with a volume ratio of buffer to mixed antibacterial solution of 5:1, and shake at 150 rpm for 48 hours at a constant temperature of 45°C; then rinse with PBS for 1 hour; the mixed antibacterial solution is composed of penicillin and streptomycin, with a volume ratio of penicillin to streptomycin of 1:1;

（4）浓度为5%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150rpm震荡48小时；再用PBS冲洗1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]

(5) Add 10 KIU/ml and 10 g/ml mixed antibacterial solution to 0.5 mg/ml PBS buffer containing DNase. The volume ratio of the buffer to the mixed antibacterial solution is 5:1. Shake at 150 rpm for 12 hours at 37°C. Then rinse with PBS for 1 hour. The mixed antibacterial solution is composed of penicillin and streptomycin. The volume ratio of penicillin to streptomycin is 1:1.

（5）浓度为0. 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150rpm震荡12小时，再用PBS冲洗1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]

The main advantages of this invention are as follows:

本发明的主要优点如下：

[0016]

(1) The whole intervertebral disc used in this invention is a naturally derived biological material with good biocompatibility and wide availability;

（1）本发明所采用的全椎间盘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；

[0017]

(2) The whole intervertebral disc obtained by decellularization technology does not contain cells or other antigens, which can minimize the immune rejection reaction of the recipient, and at the same time does not contain harmful components such as bacteria and viruses, thus having high biosafety.

（2）采用脱细胞技术获得的全椎间盘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；

[0018]

(3) 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 components and structure of normal intervertebral discs to the greatest extent.

（3）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椎间盘成分和结构；

[0019]

(4) 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 it can also be ground into powder to dissolve the biochemical factors contained in normal intervertebral discs for the treatment of degenerated intervertebral discs.

（4）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。

[0020]

Attached Figure Description

附图说明

[0021]

Figure 1 is a gross external view of the entire intervertebral disc (annulus fibrosus and nucleus pulposus) according to the present invention;

图1是本发明的全椎间盘（纤维环和髓核）大体外观图；

[0022]

Figure 2 shows the HE staining of the annulus fibrosus scaffold, which is cell-free and free of residual nuclear components.

图2是纤维环支架的HE染色无细胞及无细胞核成分残留图；

[0023]

Figure 3 shows HE staining of the nucleus pulposus scaffold, revealing cell-free and cell-free nuclear component residues.

图3是髓核支架的HE染色无细胞及无细胞核成分残留图；

[0024]

Figure 4 shows the fibrous ring scaffold retaining a large amount of glycosaminoglycan components after Alcian blue staining.

图4是纤维环支架的阿尔新蓝染色保留大量糖胺聚糖成分图；

[0025]

Figure 5 shows the Alcian blue staining of the nucleus pulposus scaffold, which retains a large amount of glycosaminoglycan components.

图5是髓核支架的阿尔新蓝染色保留大量糖胺聚糖成分图；

[0026]

Figure 6 shows that the whole intervertebral disc DNA quantification test contained almost no DNA components;

图6是全椎间盘DNA定量检测几乎不含有DNA成分图；

[0027]

Figure 7 shows the intact preservation of collagen fiber arrangement and three-dimensional spatial structure as detected by scanning electron microscopy of the annulus fibrosus.

图7是纤维环扫描电镜检测胶原纤维排布和空间立体结构完整保留图；

[0028]

Figure 8 shows the intact preservation of collagen fiber arrangement and three-dimensional spatial structure in the nucleus pulposus as detected by scanning electron microscopy.

图8是髓核扫描电镜检测胶原纤维排布和空间立体结构完整保留图；

[0029]

Figure 9 shows the proliferation of MSCs under different scaffold extract concentrations as detected by CCK-8.

图9是CCK-8检测不同支架浸提液浓度下MSCs的增值情况图；

[0030]

Figure 10 shows the growth of Live/Dead cell-stained MSCs on a whole intervertebral disc decellularized scaffold.

图10是Live/Dead细胞染色MSCs在全椎间盘脱细胞支架上的生长情况图。

[0031]

Detailed Implementation Plan

具体实施方案

[0032]

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。

[0033]

1. Preparation of decellularized matrix of whole intervertebral disc

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1、制备全椎间盘脱细胞基质

[0034]

(1) Material collection: Take the intervertebral discs from T5 to L3 of healthy rabbits that are 4 months old (male or female), remove the bony fragments, rinse thoroughly with sterile PBS to remove blood and other impurities. The long axis of the intervertebral disc is about 1cm, the short axis is 0.8cm, and the thickness is about 5mm.

（1）取材：取4个月（雌雄均可）健康家兔胸5到腰3的椎间盘，摘除骨性碎片，无菌PBS充分漂洗去除血液和其他杂质，椎间盘长轴约1cm，短轴0.8cm，厚度约5mm。

[0035]

The decellularization steps are as follows:

脱细胞步骤如下：

[0036]

Step 1: In 100 ml of 10% PBS buffer containing 10 KIU/ml protease inhibitor, shake at 150 rpm on a low temperature (4 ^o C) for 4 hours; then rinse with PBS for 1 hour;

步骤一:在100ml浓度为10%的含10KIU/ml 蛋白酶抑制剂的PBS缓冲液，低温（4 ^o C）摇床150rpm震荡4小时；并用PBS冲洗1小时；

[0037]

Step 2: Add 100 ml of penicillin and streptomycin (10 KIU/ml, 10 g/ml) mixed antibacterial solution to 500 ml of 4% Triton X-100 PBS buffer, and shake at 150 rpm on a low temperature (4 ^o C) for 48 hours; then rinse with PBS for 1 hour;

步骤二:在500ml浓度为4%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，低温（4 ^o C）摇床150rpm震荡48小时；并用PBS冲洗1小时；

[0038]

Step 3: Add 100 ml of penicillin and streptomycin (10 KIU/ml, 10 g/ml) to 500 ml of 5% SDS-containing PBS buffer and mix with a shaker at 150 rpm for 48 hours at low temperature (4 NER 7 °C); then rinse with PBS for 1 hour.

步骤三:在500ml浓度为5%的含SDS的PBS缓冲液，加入100ml青霉素和链霉素（10KIU/ml，10g/ml）混合抗菌液，低温（4 ^o C）摇床150rpm震荡48小时；并用PBS冲洗1小时；

[0039]

Step 4: Add 20 ml of penicillin and streptomycin (10 KIU/ml, 10 g/ml) mixed antibacterial solution to 100 ml of 0.5 mg/ml PBS buffer containing DNase. Shake at 150 rpm for 12 hours at 37°C. Then wash with PBS for 1 hour to obtain a whole intervertebral disc decellularized scaffold, as shown in Figure 1.

步骤四：用100ml浓度为0. 5mg/ml的含DNA酶的PBS缓冲液，加入20ml青霉素和链霉素（10KIU/ml，10g/ml）混合抗菌液，37 ^o C摇床150rpm震荡12小时后，并用PBS冲洗1小时得到全椎间盘脱细胞支架，如图1所示；

[0040]

Histological evaluation of whole intervertebral disc decellularized scaffold

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全椎间盘脱细胞支架的组织学评价

[0041]

Figures 2 and 3 are 100x magnification images of the decellularized intervertebral disc scaffold (annulus fibrosus and nucleus pulposus) of the present invention after HE staining, showing no residual cells or nuclei; Figures 4 and 5 are 100x magnification images of the decellularized intervertebral disc scaffold (annulus fibrosus and nucleus pulposus) of the present invention after Alcian blue staining, showing the retention of a large amount of glycosaminoglycans.

图2和图3是本发明全椎间盘脱细胞支架（纤维环和髓核）HE染色放大100倍无细胞及无细胞核成分残留图；图4和图5是本发明全椎间盘脱细胞支架（纤维环和髓核）阿尔新蓝染色放大100倍保留大量糖胺聚糖成分图。

[0042]

(4) Quantitative detection of antigen components in whole intervertebral disc decellularized scaffold

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（4）全椎间盘脱细胞支架的抗原成分定量检测

[0043]

Figure 6 shows that the DNA quantification of the whole intervertebral disc decellularized scaffold (annulus fibrosus and nucleus pulposus) of the present invention contains almost no DNA components.

图6是本发明全椎间盘脱细胞支架（纤维环和髓核）DNA定量检测几乎不含有DNA成分图。

[0044]

(5) Observation of the ultrastructure of the whole intervertebral disc decellularized scaffold

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（5）全椎间盘脱细胞支架的超微立体结构观察

[0045]

Figures 7 and 8 are scanning electron microscopy images of the collagen fiber arrangement and complete preservation of the three-dimensional spatial structure of the whole intervertebral disc decellularized scaffold (annulus fibrosus and nucleus pulposus) of the present invention.

图7和图8是本发明全椎间盘脱细胞支架（纤维环和髓核）扫描电镜检测胶原纤维排布和空间立体结构完整保留图。

[0046]

(6) Biocompatibility evaluation of whole intervertebral disc decellularized scaffold

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（6）全椎间盘脱细胞支架的生物相容性评价

[0047]

Figure 9 shows the proliferation of MSCs under different scaffold extract concentrations detected by the whole intervertebral disc decellularized scaffold CCK-8 of the present invention.

图9是本发明全椎间盘脱细胞支架CCK-8检测不同支架浸提液浓度下MSCs的增值情况图。

Figure 10 shows the growth of Live/Dead cell-stained MSCs on the whole intervertebral disc decellularized scaffold of the present invention at 100x magnification.

图10是本发明全椎间盘脱细胞支架Live/Dead细胞染色MSCs在全椎间盘脱细胞支架上100倍的生长情况图。