Notice

DESCRIPTION CN110237303A

Preparation method of decellularized periosteum matrix gel material from natural tissue

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天然组织来源的脱细胞骨膜基质凝胶材料的制备方法

[0001]

Technical Field

技术领域

[0002]

This invention belongs to the field of bone tissue repair and regeneration technology, specifically relating to a method for preparing decellularized periosteal matrix gel materials derived from natural tissues.

本发明属于骨组织修复及其再生技术领域，具体涉及天然组织来源的脱细胞骨膜基质凝胶材料的制备方法。

[0003]

Background Technology

背景技术

[0004]

Bone defects are a common clinical condition that often leads to nonunion, delayed healing or nonunion, and local functional impairment, causing pain, limiting mobility, and severely impacting quality of life for patients.

骨缺损是临床常见疾病，往往会造成骨不连接、延迟愈合或不愈合及局部功能障碍，对患者造成痛苦，导致其行动受限，生活质量受到严重影响。

While traditional synthetic materials can provide sufficient mechanical support, they have poor bioactivity, insufficient osteoinductive properties, are not easily degraded, and may even cause foreign body reactions.

传统人工合成材料虽然能提供足够的力学支撑，但其生物活性差，骨诱导性不足，不易降解，甚至引起异物反应。

Currently, the main methods for treating bone defects and osteonecrosis are autologous bone transplantation and allogeneic bone transplantation. However, autologous bone transplantation can cause secondary damage, while allogeneic bone transplantation carries potential risks of immune and disease transmission. Therefore, there is an urgent need to develop new bone tissue engineering materials with good bone healing, regeneration and repair effects.

目前，治疗骨缺损和骨坏死等的方法以自体骨移植和同种异体骨移植为主，但自体骨移植会产生二次损伤，而同种异体骨移植因存在潜在免疫和疫病传播风险，因此亟需研发新的具有良好骨愈合再生修复效果的骨组织工程材料。

[0005]

The extracellular matrix (ECM) is a macromolecule synthesized and secreted by cells outside the cell. Its main components include collagen fibers, glycoproteins, polysaccharides, and growth factors.

细胞外基质(Extracellular matrix,ECM)是由细胞合成并分泌到胞外的大分子，主要成分包括胶原纤维、糖蛋白、多聚糖、生长因子等。

ECM forms a complex network structure that supports and connects tissue structures and regulates a series of physiological activities such as tissue cell growth.

ECM构成复杂的网架结构，支持并连接组织结构，调节组织细胞的生长等系列生理活动。

Hydrogels are a class of scaffold materials that have attracted much attention in tissue engineering, with advantages such as injectability, minimal damage, and arbitrary shaping. Common hydrogel products are composed of a single or a few specific ingredients, making it difficult to perfectly mimic the components of biological tissues. If additional growth factors or active molecules are loaded, there is a risk of side effects and tumorigenesis. Patent application number 201410145791.7 discloses a decellularized matrix gel and a new method for its preparation. After decellularization and digestion, the gel is incubated at a lower temperature to reduce damage to the tissue. Patent application number 201811452768.7 discloses a method for preparing decellularized matrix gel. In addition to using a mild

decellularization method, the decellularized matrix gel is also processed under pressure after digestion, which achieves better repair effect.

水凝胶是组织工程中一类较受关注的支架材料体系，具有可注射，损伤小，任意塑形等优点。 而常见的水凝胶产品有单一或少数特定成分构成，难以完美模拟生物组织成分，若负载另外的生长因子或活性分子，有产生副效应和致瘤等风险。 申请号为201410145791.7的专利公开了一种脱细胞基质凝胶及其制备新方法，脱细胞、消化后通过较低温恒温孵育减少了对组织的损伤。 申请号为201811452768.7的专利公开了一种脱细胞基质凝胶制备的方法，除了采用温和的脱细胞法，还在脱细胞基质凝胶消化后，在加压条件下进行处理，获得了较好修复效果。

[0006]

However, the previous preparation of decellularized matrix gel materials by cutting and shredding could not obtain sufficient specific surface area, which was not conducive to sufficient contact with digestive fluids and resulted in low retention of bioactive substances.

但既往脱细胞基质凝胶材料的制备通过剪碎方式无法获得足够的比表面积，不利于消化液的充分接触，生物活性物质保留低。

Furthermore, there are currently no standardized products for the preparation of periosteal ECM gel. The periosteum is an important component of bone. The tissue specificity of the periosteum ECM is more conducive to bone regeneration and repair, and it cannot be

replaced by other ectopic tissues as repair materials. Therefore, based on the above background, this patent proposes a method to achieve maximum retention of active molecules of periosteal ECM through freeze-drying; and to increase the contact area of the solution by grinding ECM into powder; and to control the particle size of the powder by sieve filtration, thus standardizing the digestion steps. This results in a periosteal ECM gel repair material with high bioactivity retention and standardized specifications. In addition, EDC cross-linked growth factors have a good promoting effect on achieving excellent regeneration and vascularization of new bone tissue. It can be used to repair various bone regeneration disorders such as bone defects and nonunion caused by various etiologies in clinical practice.

此外，目前并未有骨膜ECM凝胶标准化制备的产品。 骨膜是骨的重要组成部分，骨膜ECM的组织特异性更有利于骨的再生修复，是其他异位组织作为修复材料所无法取代的。 因此，基于上述背景，本专利提出一种通过冷冻干燥的方案实现对骨膜ECM活性分子的最大保留；并且将ECM研磨成粉增加溶液接触面积；通过筛网过滤控制粉末粒径，标化消化步骤。 从而得到具有生物活性高保留、标准统一化的骨膜ECM凝胶修复材料。 此外，通过EDC交联生长因子，对实现新生骨组织的优异再生、血管化长入具有很好的促进效果。 可用于修复临床上各类病因造成的骨缺损，骨不愈合等多种骨再生障碍问题。

[0007]

Summary of the Invention

发明内容

[0008]

In view of this, the purpose of the present invention is to provide a decellularized periosteum matrix gel material with high retention of bioactive substances and a method for preparing the same.

有鉴于此，本发明的目的在于提供一种生物活性物质高保留的脱细胞骨膜基质凝胶材料及其制备方法。

[0009]

To achieve the above-mentioned objectives, the present invention provides the following technical solution: after decellularization of periosteum tissue derived from natural tissue, highly active periosteum ECM gel material is obtained through freeze-drying, grinding, and gel treatment.

为了实现上述发明目的，本发明提供以下技术方案：对天然组织来源的骨膜组织经脱细胞处理后，通过冷冻干燥、磨粉、凝胶处理，得到高活性的骨膜ECM凝胶材料。

Furthermore, by using modified gel technology, bioactive factors (VEGF, FGF) are introduced and grafted onto the gel collagen fiber network to achieve a combination of specific functions.

并通过修饰胶的技术，引入生物活性因子(VEGF、FGF)，将其接枝于凝胶胶原纤维网络，实现特定功能的组合。

[0010]

The naturally derived periosteal tissue includes mammalian bone tissue, specifically the periosteum of the long bones of the limbs and the periosteum of the skull; the decellularization technique comprises a decellularization scheme using chemical reagents such as protease inhibitors, KCl, KI, acetone, tributyl phosphate, Triton X-100, and sodium dodecyl sulfate (SDS), combined with a physical freeze-thaw method, and specifically includes the following steps:

所述的天然组织来源的骨膜组织包括哺乳动物骨组织骨膜为四肢长骨骨膜以及颅骨骨膜；所述的脱细胞技术包括蛋白酶抑制剂、KCl、KI、丙酮、磷酸三丁酯、Triton X-100、十二烷基硫酸钠(SDS)等化学试剂和物理冻融法组成的脱细胞方案，具体包括以下步骤：

[0011]

a) The muscles, fascia, and fat surrounding the periosteum are initially removed and used as raw materials;

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a)将初步去除骨膜周边肌肉、筋膜和脂肪作为原材料；

[0012]

b) Rinse the periosteal tissue material three times with sterile water for 20 minutes each time to remove residual muscle, fascia and fat, and irradiate it with 24kGy dose of gamma rays for 24 hours to kill any bacteria and viruses present.

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b)将骨膜组织材料用无菌水漂洗3次，每次20分钟，去除残余的肌肉、筋膜和脂肪，用24kGy剂量的γ射线照射24h，杀灭存在的细菌和病毒；

[0013]

c) Treat the material obtained in b) with tributyl phosphate and deionized water for 12-24 hours; the volume ratio of tributyl phosphate to deionized water is 1%-5%.

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c)将b)得到的材料经磷酸三丁酯与去离子水处理12-24小时；磷酸三丁酯与去离子水的体积比为1%-5%；

[0014]

d) Immerse the material obtained in c) in a 1% Triton X-100 solution and shake at 200 rpm for 12 hours at a constant temperature of 23°C. Rinse with sterile water 3 times for 20 minutes each time to remove residual Triton X-100 solution.

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d)将c)得到的材料浸泡于浓度为1%TritonX-100溶液中，恒温23°C摇床200rpm震荡12小时，用无菌水漂洗3次，每次20分钟，去除残留TritonX-100溶液；

[0015]

e) Immerse the material obtained in d) in a 1% SDS solution, shake at 200 rpm for 2 hours at a constant temperature of 23°C, and rinse with ddH₂O for 12 hours to remove residual SDS solution.

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e)将d)得到的材料浸泡于摩尔浓度为1%SDS溶液中，恒温23°C摇床200rpm震荡2小时，用ddH₂O流动冲洗12小时，去除残留SDS溶液；

[0016]

f) Immerse the material obtained in e) in PBS buffer containing KCl for 8 hours. The concentration of KCl is 2M.

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f)将e)得到的材料浸泡于含KCl的PBS缓冲液中8小时，KCl的浓度为2M；

[0017]

g) Soak the material obtained in f) in 2M KI-containing PBS buffer for 10 hours, and then rinse with ddH₂O for 12 hours to remove residual PBS buffer.

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g)将f)得到的材料浸泡于含KI的PBS缓冲液中10小时，KI的浓度为2M，ddH₂O流动冲洗12小时去除残留PBS缓冲液。

[0018]

h) Sterilize the material obtained in g) by irradiation;

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h)将g)得到的材料辐照灭菌；

[0019]

The material obtained in g) was irradiated with gamma rays for 10-48 hours to kill any bacteria and viruses that may be present.

将g)得到的材料经γ射线照射10-48小时，杀灭可能存在的细菌和病毒；

[0020]

i) Freeze-dry the material obtained in h);

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i)将h)得到的材料冷冻干燥；

[0021]

After sterilization, the materials are first pre-cooled in a -80°C refrigerator, and then dried in a freeze dryer for 24 hours, maintaining a vacuum of 1.5 mtorr and a temperature of -50°C.

将灭菌后的材料先放入-80°C冰箱预冷后，放入冷冻干燥机干燥24小时，保持真空度为1.5mtorr，温度-50°C。

[0022]

j) Grind the material obtained in i) into powder.

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j)将i)得到的材料磨粉

[0023]

The freeze-dried material was transferred to a grinder at a frequency of 60 Hz for 1 minute. After being frozen with liquid nitrogen, the material was ground into powder and passed through a 40-100 mesh sieve to collect the freeze-dried tissue powder.

将冻干的材料转移到研磨器中，研磨机频率为60HZ，设定时间1分钟，经液氮冷冻，研磨成粉末，过40-100目筛网，收集冻干组织粉末；

[0024]

k) Gel the material obtained in j);

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k)将j)得到的材料凝胶；

[0025]

The powder obtained in j) was soaked in glacial acetic acid solution, pepsin was added, and digestion was carried out at room temperature for 24-72 hours. After complete digestion, the solution was centrifuged at 500×g for 10 min to remove impurities. The pH was then adjusted to about 7.4 with NaOH solution to obtain the periosteal ECM gel material, which was stored at 4°C.

将将j)得到的粉末浸泡于冰醋酸溶液中，加入胃蛋白酶，常温消化24-72小时，待完全消化，溶液500×g离心10min，去除杂质后用NaOH溶液调节pH至7.4左右，即得骨膜ECM凝胶材料，4℃保存。

[0026]

The obtained periosteal ECM gel material was cross-linked with EDC and NHS to increase the cross-linking degree of the gel fiber network.

将所得骨膜ECM凝胶材料使用交联剂增加凝胶纤维网络的交联度，交联剂为EDC和NHS。

[0027]

Preferably, the irradiation dose in h) is 5-40 kGy.

作为优选，h)中的辐照剂量为5-40kGy。

[0028]

Preferably, the concentration of the glacial acetic acid is 0.3-0.7M.

作为优选，所述的冰醋酸浓度为0.3-0.7M。

[0029]

As a preferred option, tissue particles are sieved through an 80-mesh sieve.

作为优选，选择80目筛网筛过组织颗粒。

[0030]

Preferably, the amount of pepsin added is 2-20% of the total solution.

作为优选，胃蛋白酶加入量为占总溶液的2-20%。

[0031]

Preferably, EDC is used as a crosslinking agent with a volume concentration of 0.1-0.4%, and the EDC:NHS ratio can be 2:1, 3:1, 3:2, 4:3, or 5:3.

作为优选，EDC为交联剂，体积浓度为0.1-0.4%，EDC：NHS比例可为2:1，3:1，3:2，4:3，5:3。

[0032]

Preferably, bioactive factors are introduced and grafted onto the gel collagen fiber network. The bioactive factors are VEGF or FGF.

作为优选，引入生物活性因子接枝于凝胶胶原纤维网络，生物活性因子为VEGF或FGF。

[0033]

The beneficial effects of this invention are:

本发明的有益效果：

[0034]

The natural tissue-derived periosteal ECM gel of the present invention can retain a variety of bioactive molecules in the matrix, and the gel particles are standardized in size. Furthermore, through EDC cross-linking of various bioactive factors, it can achieve excellent regeneration of new bone tissue and has a good promoting effect on vascularization ingrowth.

本发明的天然组织来源的骨膜ECM凝胶能够高保留基质中多种生物活性分子，且凝胶颗粒尺寸标准化，并且通过EDC交联多种生物活性因子，能够实现新生骨组织的优异再生、血管化长入具有很好的促进效果。

[0035]

Compared to existing technologies for treating gels or types of gels, the significant advancement of this invention lies in:

相比现有技术处理的凝胶或凝胶种类，本发明显著的进步在于：

[0036]

1) Using mammalian periosteum as raw material, the previous method of cutting and shredding could not obtain sufficient specific surface area, which was not conducive to the full contact of digestive juices; this patent increases digestion efficiency by freeze-drying and grinding.

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1)以哺乳动物骨膜为原材料，既往通过剪碎方式无法获得足够的比表面积，不利于消化液的充分接触；本专利通过冻干磨粉处理，增加了消化效率。

[0037]

2) In the past, the digestion of tissue particles was carried out after grinding without controlling the particle size, which was not conducive to the standardization and repeatability of the digestion process.

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2)既往通过研磨后组织颗粒即进行消化，未标控颗粒尺寸，不利于消化流程的标准化和重复性。

This patent utilizes an improved digestion control technology to facilitate the stable production of hydrogels with controllable quality.

本专利通过改良的消化控制技术，有利于稳定产生品质可控的水凝胶。

[0038]

3) The technology of this invention improves the retention efficiency of active molecules such as collagen, GAGs and various growth factors in the natural matrix, so as to exert effects on cell activity, proliferation, migration, morphology and differentiation, thereby promoting in situ regeneration of functional tissues and having good biological activity.

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3)本发明技术提高了对天然基质中的胶原、GAGs和多种生长因子等活性分子的保留效率，以发挥影响细胞活性、增殖、迁移、形态和分化等作用，进而促进功能组织的原位再生，具有良好的生物学活性。

[0039]

4) The periosteal ECM gel of the present invention crosslinks multiple growth factors, such as VEGF and FGF, through EDC, thereby increasing the repair function of the periosteal ECM gel material.

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4)本发明的骨膜ECM凝胶通过EDC交联多种生长因子，如VEGF、FGF等，增加了骨膜ECM凝胶材料的修复功能。

Compared to the side effects of abnormal calcification caused by current aldehyde crosslinking agents, EDC crosslinking agents have better biosafety and can be completely eliminated after catalytic reaction.

相比目前醛类交联剂引起异常钙化的副作用，EDC交联剂更好的生物安全性，且催化反应后可被完全清除。

[0040]

5) As an important tissue component of bone, the periosteum has tissue specificity that is more conducive to bone regeneration and repair, and has strong osteoinductive properties.

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5)骨膜作为骨的重要组织成分，其组织特异性更有利于骨的再生修复，骨诱导性强。

Therefore, developing high-quality periosteal ECM gel repair materials is of great significance for bone defect repair.

因此，开发品质可控的骨膜ECM凝胶修复材料，对于骨缺损修复的意义重大。

[0041]

Attached Figure Description

附图说明

[0042]

Figure 1. Schematic diagram of periosteal ECM powder, gel solution and gel material derived from natural tissue.

图1天然组织来源的骨膜ECM粉末、凝胶溶液及凝胶材料示意图。

[0043]

Figure 2 shows HE and DAPI staining of the periosteal ECM from natural tissue, demonstrating that the DNA residue in this material is extremely low.

图2天然组织来源的骨膜ECM的HE染色和DAPI染色，证明本材料DNA残留量极低。

[0044]

Figure 3. Scanning electron microscopy observation of type I collagen gel (8 mg/ml), periosteal ECM gel (4 mg/ml), and type I collagen gel (8 mg/ml).

图3 I 型胶原凝胶8mg/ml、骨膜ECM凝胶4mg/ml和8mg/ml扫描电镜观察。

Under a microscopic scale, periosteal ECM gel has a nanofiber network that is highly similar to type I collagen gel, and this structure has a good promoting effect on tissue repair.

骨膜ECM凝胶微观下同I型胶原凝胶有着高度相似的纳米纤维网络，这种结构对于组织修复有着很好的促进作用。

[0045]

Figure 4. In vitro biocompatibility assessment of the periosteal ECM gel material derived from natural tissue.

图4天然组织来源的骨膜ECM凝胶材料体外生物相容性的评估。

(a) Mouse bone marrow mesenchymal stem cells (mBMSCs) showed good cell viability after 1, 3 and 7 days of culture on periosteal ECM gel, indicating the safety and good growth-promoting properties of periosteal ECM gel.

(a)鼠骨髓间充质干细胞(mBMSCs)在骨膜ECM凝胶上培养1、3、7天的细胞活性良好，说明骨膜ECM凝胶安全性及良好的促生长性。

(b) The number of live mBMSCs increased with the number of days during periosteal ECM gel culture.

(b)mBMSCs在骨膜ECM凝胶培养过程中活细胞的数量随着天数增加。

(c) The CCK8 assay also demonstrated that mBMSCs cultured on periosteal ECM gel had good activity.

(c)CCK8法同样证明骨膜ECM凝胶上培养的mBMSCs活性良好。

[0046]

Figure 5. Evaluation of the angiogenic activity of the periosteum ECM gel material derived from natural tissue.

图5天然组织来源的骨膜ECM凝胶材料血管生成活性的评价。

In in vitro experiments on the formation of endothelial cell tubes, type I collagen and periosteal ECM gel materials showed that periosteal ECM gel material exhibited stronger pro-angiogenic activity.

I型胶原和骨膜ECM凝胶材料在体外内皮细胞管样的形成实验中，骨膜ECM凝胶材料展现出更强的促血管生成活性。

[0047]

Figure 6. Evaluation of spontaneous mineralization of periosteal ECM gel material derived from natural tissue in vitro.

图6天然组织来源的骨膜ECM凝胶材料在体外自发矿化的评价。

(a, b, c) Micro-CT scans of periosteal ECM gel material immersed in m-SBF (simulated human body fluid). Periosteal ECM gel has a higher mineral content than type I collagen.

(a，b，c)骨膜ECM凝胶材料浸泡在m-SBF(模拟人体液)中的micro-CT扫描图，骨膜ECM凝胶比I型胶原有着更高的矿物含量骨膜ECM。

(d) The periosteal ECM gel material showed more mineralized nodules under Von Kossa staining, larger diameter mineralized fibers under scanning electron microscopy, and more prominent mineralized diffraction rings under single-crystal transmission electron microscopy.

(d)骨膜ECM凝胶材料在Von Kossa染色下有更丰富的矿化结节、扫描电镜下有更大直径的矿化纤维，透射电镜单晶衍射下更显著的矿化衍射环。

This indicates that the periosteal gel scaffold provides more active sites, showing a higher level of calcium and phosphorus deposition, which is conducive to spontaneous mineralization.

说明骨膜凝胶支架提供更丰富的活性位点，显示出更高的钙磷沉积水平，有利于自发矿化。

[0048]

Figure 7 Evaluation of osteogenic activity of primary cranial cells by periosteal ECM gel material derived from natural tissues.

图7天然组织来源的骨膜ECM凝胶材料对原代颅骨细胞成骨活性的评价。

(a) Relative expression of Runx 2, ALP, OPN and COL 1α when osteoblasts were grown on 8 mg /ml type I collagen and PEM hydrogel for 14 days.

(a)成骨细胞在8mg/ml Ⅰ型胶原和PEM水凝胶上生长14天时，Runx 2、ALP、OPN、COL 1α的相对表达。

(b) Expression and location of Runx 2 and OCN in osteoblasts grown on 8 mg/ml type I collagen and PEM hydrogel for 14 and 28 days.

(b)成骨细胞在8mg/ml Ⅰ型胶原和PEM水凝胶上生长14和28天时，Runx 2和OCN在细胞内的表达和位置。

This indicates that osteoblasts cultured on periosteal ECM gel scaffolds express higher levels of osteogenic markers.

表明骨膜ECM凝胶支架上培养的成骨细胞表达更高水平的成骨标志物。

[0049]

Figure 8. Results of in vivo cranial bone formation experiments using periosteum ECM gel material derived from natural tissue.

图8天然组织来源的骨膜ECM凝胶材料体内颅骨形成实验结果。

Periosteal ECM gel material promotes more new bone formation at skull defects.

骨膜ECM凝胶材料促进颅骨缺损部位更多的新骨形成。

[0050]

Figure 9. Subcutaneous embedding results of periosteal ECM gel material derived from natural tissue.

图9天然组织来源的骨膜ECM凝胶材料皮下包埋结果。

(a) Gross images of subcutaneously implanted gels (8 mg/ml type I collagen hydrogel, 4 mg /ml periosteal hydrogel, 8 mg/ml periosteal hydrogel) at 3, 7 and 21 days, showing a stable degradation trend.

(a)皮下植入凝胶(8mg/ml Ⅰ型胶原水凝胶，4mg/ml骨膜水凝胶，8mg/ml骨膜水凝胶)3、7、21d的大体图像，呈现稳定降解趋势。

(b) Histological evaluation of the systemic organ toxicity of subcutaneously implanted hydrogels showed no obvious organ toxicity.

(b)皮下植入水凝胶对全身器官毒性损伤的组织学评价，未显现出明显的脏器毒害反应。

(c) Histological images of subcutaneous hydrogel implantation on days 3, 7, and 21. Periosteal ECM gel showed a low immune response and rapid regression, promoting angiogenesis and migration into the scaffold.

(c)第3、7、21天皮下植入水凝胶的组织学图像，骨膜ECM凝胶表现出较低的免疫反应并迅速消退，促进血管生成和迁入支架内部

[0051]

Detailed Implementation

具体实施方式

[0052]

The following detailed description, in conjunction with embodiments, illustrates a naturally derived decellularized periosteal ECM gel material and its preparation method provided by the present invention. However, these descriptions should not be construed as limiting the scope of protection of the present invention.

下面结合实施例对本发明提供的一种天然组织来源的脱细胞骨膜ECM凝胶材料及其制备方法进行详细的说明，但是不能把它们理解为对本发明保护范围的限定。

[0053]

Example 1: Preparation of femoral periosteum ECM gel derived from natural tissue

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实施例1天然组织来源的股骨骨膜ECM凝胶的制备

[0054]

1. Use a blade to remove the muscles, fascia, and fat from the femur of an adult pig, and cut the periosteum of the femur as raw material;

1、用刀片剔除成年猪股骨上的肌肉、筋膜和脂肪等组织，切取股骨骨膜作为原材料；

[0055]

2. Rinse the material obtained in step 1 with sterile water 3 times, 20 minutes each time, to remove residual muscle, fascia and fat tissues, and irradiate with 24kGy dose of γ rays for 24 hours to kill any bacteria and viruses that may exist.

2、将步骤1所得的材料用无菌水漂洗3次，每次20分钟，去除残余的肌肉、筋膜和脂肪等组织，用24kGy剂量的γ射线照射24h，杀灭可能存在的细菌和病毒；

[0056]

3. The material obtained in step 2 is treated with tributyl phosphate and deionized water at a volume ratio of 2% for 24 hours;

3、将步骤2所得的材料经磷酸三丁酯与去离子水的体积比为2%处理24小时；

[0057]

4. Immerse the material obtained in step 3 in a 1% Triton X-100 solution and shake it at 200 rpm for 12 hours at a constant temperature of 23°C. Rinse it three times with sterile water for 20 minutes each time to remove residual Triton X-100 solution.

4、将步骤3所得的材料浸泡于浓度1%TritonX-100溶液中，恒温23°C摇床200rpm震荡12小时，用无菌水漂洗3次，每次20分钟，去除残留TritonX-100溶液；

[0058]

5. Immerse the material obtained in step 4 in a 1% SDS solution, shake at 200 rpm for 2 hours at a constant temperature of 23°C, and rinse with ddH₂O for 12 hours to remove residual SDS solution.

5、将步骤4所得的材料浸泡于浓度1%SDS溶液中，恒温23°C摇床200rpm震荡2小时，用ddH₂O流动冲洗12小时，去除残留SDS溶液；

[0059]

6. Soak the material obtained in step 5 in PBS buffer containing KCl for 8 hours. The concentration of KCl is 2M.

6、将步骤5所得的材料浸泡于含KCl的PBS缓冲液中8小时，KCl的浓度为2M；

[0060]

7. Soak the material obtained in step 6 in PBS buffer containing KI for 10 hours. The concentration of KI is 2M. Rinse with ddH₂O for 12 hours to remove residual PBS buffer.

7、将步骤6所得的材料浸泡于含KI的PBS缓冲液中10小时，KI的浓度为2M，ddH₂O流动冲洗12小时去除残留PBS缓冲液。

[0061]

8. Irradiate the material obtained in step 7 with 24 kGy gamma rays for 24 hours to kill any bacteria and viruses that may be present.

8、将步骤7所得的材料用24kGy剂量的γ射线照射24小时，杀灭可能存在的细菌和病毒；

[0062]

9. After pre-cooling the sterilized decellularized periosteum material obtained in step 8 in a freezer at -80°C, place it in a freeze dryer and dry for 24 hours, maintaining a vacuum of 1.5 mtorr, a temperature of -50°C, and a vacuum pump pumping rate of 195 L/min at 60 Hz.

9、将步骤8所得的灭菌脱细胞骨膜材料在-80°C冰箱预冷后，放入冷冻干燥机干燥24小时，保持真空度为1.5mtorr，温度-50°C，真空泵抽气速率195L/min，60Hz；

[0063]

The freeze-dried material obtained in step 9 was transferred to a grinder with a grinder frequency of 60 Hz and a set time of 1 minute. After being frozen with liquid nitrogen, it was ground into powder, passed through a 100-mesh sieve, and the freeze-dried tissue powder was collected. The obtained particle diameter was about 150 μm.

将步骤9所得的冻干材料转移到研磨器中，研磨机频率为60HZ，设定时间1分钟，经液氮冷冻，研磨成粉末，过100目筛网，收集冻干组织粉末，所获颗粒直径约150μm；

[0064]

10. Soak the powder obtained in step 10 in 0.5M glacial acetic acid solution to prepare a bone membrane ECM powder suspension containing 10% pepsin, and digest it at room temperature for 48 hours.

10、将步骤10所得的粉末浸泡于0.5M冰醋酸溶液中，制成含有10%胃蛋白酶的骨膜ECM粉末悬液，常温消化48小时；

[0065]

11. Centrifuge the suspension obtained in step 11 at 500×g for 10min to remove impurities, and then adjust the pH to about 7.4 with NaOH solution to obtain the periosteal ECM gel material, which is stored at 4°C.

11、将步骤11所得的悬液500×g离心10min，去除杂质后用NaOH溶液调节pH至7.4左右，即得骨膜ECM凝胶材料，4°C保存。

As shown in Figure 1.

如图1所示。

[0066]

Example 2: Preparation of scapular periosteum ECM gel derived from natural tissue

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实施例2天然组织来源的肩胛骨骨膜ECM凝胶的制备

[0067]

1. Use a blade to remove the muscles, fascia, and fat from the scapula of an adult pig, and cut the scapular periosteum as raw material;

1、用刀片剔除成年猪肩胛骨上的肌肉、筋膜和脂肪等组织，切取肩胛骨骨膜作为原材料；

[0068]

2. The decellularization process is the same as steps 2-7 in Example 1 above; wherein the volume ratio of tributyl phosphate to deionized water in step 3 is 1%; and the processing time is 12 hours.

2、脱细胞处理步骤同上述实施例1中步骤2-7；其中步骤3中的磷酸三丁酯与去离子水的体积比为1%；处理时间为12小时；

[0069]

3. Irradiate the material obtained in step 7 with 40 kGy of gamma rays for 10 hours to kill any bacteria and viruses that may be present.

3、将步骤7所得的材料用40kGy剂量的γ射线照射10小时，杀灭可能存在的细菌和病毒；

[0070]

4. After pre-cooling the sterilized decellularized periosteum material obtained in step 8 in a freezer at -80°C, place it in a freeze dryer and dry for 24 hours, maintaining a vacuum of 1.5 mtorr, a temperature of -50°C, and a vacuum pump pumping rate of 195 L/min at 60 Hz.

4、将步骤8所得的灭菌脱细胞骨膜材料在-80°C冰箱预冷后，放入冷冻干燥机干燥24小时，保持真空度为1.5mtorr，温度-50°C，真空泵抽气速率195L/min，60Hz；

[0071]

5. Transfer the freeze-dried material obtained in step 9 to a grinder. The grinder frequency is 60 Hz and the time is set to 1 minute. After freezing with liquid nitrogen, grind it into powder, pass it through an 80-mesh sieve, and collect the freeze-dried tissue powder.

5、将步骤9所得的冻干材料转移到研磨器中，研磨机频率为60HZ，设定时间1分钟，经液氮冷冻，研磨成粉末，过80目筛网，收集冻干组织粉末；

[0072]

6. Soak the powder obtained in step 10 in 0.3M glacial acetic acid solution to prepare an ECM powder suspension containing 2% pepsin, and digest it at room temperature for 24 hours.

6、将步骤10所得的粉末浸泡于0.3M冰醋酸溶液中，制成含有2%胃蛋白酶的ECM粉末悬液，常温消化24小时；

[0073]

7. Centrifuge the suspension obtained in step 11 at 500×g for 10min to remove impurities, and then adjust the pH to about 7.4 with NaOH solution to obtain the periosteal ECM gel material. Store at 4°C.

7、将步骤11所得的悬液500×g离心10min，去除杂质后用NaOH溶液调节pH至7.4左右，即得骨膜ECM凝胶材料，4°C保存；

[0074]

Example 3: Preparation of femoral periosteum ECM gel derived from natural tissue

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实施例3天然组织来源的股骨骨膜ECM凝胶的制备

[0075]

1. Use a blade to remove the muscles, fascia, and fat from the femur of an adult pig, and cut the periosteum of the femur as raw material;

1、用刀片剔除成年猪股骨上的肌肉、筋膜和脂肪等组织，切取股骨骨膜作为原材料；

[0076]

8. The decellularization process is the same as steps 2-7 in Example 1 above; wherein the volume ratio of tributyl phosphate to deionized water in step 3 is 5%; and the processing time is 16 hours.

8、脱细胞处理步骤同上述实施例1中步骤2-7；其中步骤3中的磷酸三丁酯与去离子水的体积比为5%；处理时间为16小时；

[0077]

2. Irradiate the material obtained in step 7 with 5 kGy of gamma rays for 48 hours to kill any bacteria and viruses that may be present.

2、将步骤7所得的材料用5kGy剂量的γ射线照射48小时，杀灭可能存在的细菌和病毒；

[0078]

3. After pre-cooling the sterilized decellularized periosteum material obtained in step 8 in a freezer at -80°C, place it in a freeze dryer and dry for 24 hours, maintaining a vacuum of 1.5 mtorr, a temperature of -50°C, and a vacuum pump pumping rate of 195 L/min at 60 Hz.

3、将步骤8所得的灭菌脱细胞骨膜材料在-80°C冰箱预冷后，放入冷冻干燥机干燥24小时，保持真空度为1.5mtorr，温度-50°C，真空泵抽气速率195L/min，60Hz；

[0079]

4. Transfer the freeze-dried material obtained in step 9 to a grinder. The grinder frequency is 60 Hz and the time is set to 1 minute. After freezing with liquid nitrogen, grind it into powder, pass it through a 40-mesh sieve, and collect the freeze-dried tissue powder.

4、将步骤9所得的冻干材料转移到研磨器中，研磨机频率为60HZ，设定时间1分钟，经液氮冷冻，研磨成粉末，过40目筛网，收集冻干组织粉末；

[0080]

5. Soak the powder obtained in step 10 in 0.7M glacial acetic acid solution to prepare an ECM powder suspension containing 15% pepsin, and digest it at room temperature for 72 hours.

5、将步骤10所得的粉末浸泡于0.7M冰醋酸溶液中，制成含有15%胃蛋白酶的ECM粉末悬液，常温消化72小时；

[0081]

6. Centrifuge the suspension obtained in step 11 at 500×g for 10min to remove impurities, and then adjust the pH to about 7.4 with NaOH solution to obtain the periosteal ECM gel material, which is stored at 4°C.

6、将步骤11所得的悬液500×g离心10min，去除杂质后用NaOH溶液调节pH至7.4左右，即得骨膜ECM凝胶材料，4°C保存；

[0082]

Example 4: Preparation of cross-linked growth factors from femoral periosteum ECM gel derived from natural tissues

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实施例4天然组织来源的股骨骨膜ECM凝胶交联生长因子的制备

[0083]

1. The decellularization and gelation processes are the same as steps 1-11 in Example 1 above;

1、脱细胞及凝胶化处理步骤同上述实施例1中步骤1-11；

[0084]

2. Add the periosteal ECM gel material obtained in step 11 to 2-morphine ethanol sulfonate buffer (0.05M, pH 5.6) containing 0.4% EDC and 0.24% NHS (N-imine), and incubate at 37°C for 4 hours.

2、将步骤11所得的骨膜ECM凝胶材料加入含0.4%EDC和0.24%NHS(N-亚胺)的2-吗啡乙醇磺酸缓冲液(0.05M,pH5.6)，于37°C中孵育4h。

[0085]

3. Then immerse in 1×PBS and shake at 100 rpm for 6 hours.

3、随后浸没于1×PBS，100rpm匀速震荡6h。

[0086]

4. The material was then immersed in 4M NaCl and shaken at a constant speed of 100 rpm for 6 hours.

4、随后将材料浸没于4M NaCl，100rpm匀速震荡6h。

[0087]

5. Then immerse the material in ddH2O and shake it at a constant speed of 100 rpm for 6 hours. After washing it clean, set it aside for later use.

5、随后将材料浸没于ddH2O中，以100rpm匀速震荡6h，洗涤干净后备用。

[0088]

6. Add growth factor solution, 5 ng/mL VEGF (vascular endothelial growth factor) or 5 ng/mL FGF (human fibroblast growth factor), incubate at 37°C for 24 h to obtain a gel collagen fiber network of grafted growth factors, achieving a combination of specific functions.

6、加入生长因子溶液，5ng/mL VEGF(血管内皮生长因子)或5ng/mL FGF(人成纤维生长因子)，37°C孵育24h，获得接枝生长因子的凝胶胶原纤维网络，实现特定功能的组合。

[0089]

7. Obtain the periosteal ECM gel cross-linked growth factor (such as VEGF, FGF) material and store it at 4°C;

7、即得骨膜ECM凝胶交联生长因子(如VEGF,FGF)材料，4°C保存；

[0090]

Example 5: Histological evaluation of femoral periosteum ECM gel derived from natural tissues

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实施例5天然组织来源的股骨骨膜ECM凝胶的组织学评价

[0091]

1. The decellularization and gelation processes are the same as steps 1-11 in Example 1 above;

1、脱细胞及凝胶化处理步骤同上述实施例1中步骤1-11；

[0092]

2. The material obtained in step 11 was subjected to histological evaluation. The results are shown in Figure 2. HE staining showed that the decellularized femoral periosteum ECM gel structure from natural tissue was intact, the cell nuclear components were completely removed, and there were no cell debris residues. DAPI staining further showed that the cell nuclear components were negative and the antigenicity was completely removed.

2、将步骤11所得的材料进行组织学评价，结果如图2，HE染色显示天然组织来源的脱细胞股骨骨膜ECM凝胶结构完整，细胞核成分完全去除，无细胞碎片残留；DAPI染色进一步说明细胞核成分呈阴性，抗原性得到完全去除。

[0093]

Example 6 Surface properties of femoral periosteum ECM gel derived from natural tissue

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实施例6天然组织来源的股骨骨膜ECM凝胶的表面性质

[0094]

1. The decellularization and gelation processes are the same as steps 1-11 in Example 1 above;

1、脱细胞及凝胶化处理步骤同上述实施例1中步骤1-11；

[0095]

2. The periosteal ECM gel material and type I collagen gel obtained in step 11 were cured at 37° C, cut into gel particles of 5×5mm size, and then fixed overnight at 4°C in 0.1M phosphate buffer solution (PBS) at pH 7.0 and 2.5% w/v glutaraldehyde.

2、将步骤11得到的骨膜ECM凝胶材料、Ⅰ型胶原凝胶在37°C固化，切成5×5mm大小的凝胶颗粒，然后在pH7.0的0.1M磷酸盐缓冲溶液(PBS)，2.5%w/v戊二醛中4°C固定过夜。

[0096]

3. On the second day, wash the sample three times in 0.1M PBS at pH 7.0 for 15 min each time, fix it with 1% osmium tetroxide solution for 2 h, and wash it three times in 0.1M PBS at pH 7.0 for 15 min each time.

3、第二天，将样品置于pH7.0的0.1M PBS中清洗3次，每次15min，用1%四氧化锇溶液固定2h，用pH7.0的0.1M PBS中清洗3次，每次15min。

[0097]

4. Dehydrate in a series of gradient concentrations of ethanol solutions (30, 50, 70, 80, 90, 95 and 100% w/v, 20 min).

4、在一系列梯度浓度的乙醇溶液(30，50，70，80，90，95和100%w/v，20min)中脱水。

[0098]

5. Transfer the sample to a mixture of ethanol and isoaryl acetate (1:1 v/v) for 30 min, then soak in pure isoaryl acetate overnight, and dehydrate with liquid CO_NER5.

5、将样品转移到乙醇和乙酸异芳酯(1：1v/v)的混合物中30min，然后在纯乙酸异芳酯浸泡过夜，用液态CO₂脱水。

Subsequently, gold and palladium were sprayed onto the sample, and the treated sample was observed using a scanning electron microscope (TM-1000, Hitachi, Tokyo, Japan).

随后，喷上金钯，并使用扫描电镜(TM-1000，Hitachi，日本东京)对处理后的样品进行了观察。

As shown in Figure 3, the decellularized periosteum ECM gel has a highly similar microfiber arrangement to type I collagen, which is widely used in the preparation of composite materials, and there is no significant difference in pore size at the same concentration.

如图3所示，说明了脱细胞骨膜ECM凝胶与广泛应用于复合材料制备的I型胶原相比，微观纤维排布高度相似，相同浓度下，孔隙大小无显著差异。

It allows cells to grow and spread within it.

可以允许细胞在其内生长和传播。

[0099]

Example 7: Biocompatibility Analysis of Natural Tissue-Derived Femoral Periosteum ECM Gel

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实施例7天然组织来源的股骨骨膜ECM凝胶的生物相容性分析

[0100]

1. The decellularization and gelation processes are the same as steps 1-11 in Example 1 above;

1、脱细胞及凝胶化处理步骤同上述实施例1中步骤1-11；

[0101]

2. Digest and resuspend bone marrow mesenchymal stem cells (MSCs) to a cell density of 2×10 cells/mL. Add the material obtained in step 11 to a 96-well plate and add 100 µl of femoral periosteum ECM gel to each well.

2、骨髓间充质干细胞(MSCs)消化、重悬至2×10⁴个/mL细胞密度，将步骤11所得的材料加入至96孔板中，每孔加入100µl股骨骨膜ECM凝胶。

[0102]

3. Add 100 µl of cell suspension to the gel and then culture for 1, 3, and 7 days.

3、在凝胶中加入100µl细胞悬液，然后培养1、3、7天。

[0103]

4. For the cells from step 3, stain them with 1 mM live dye and 2.5 mg/ml propidium iodide (PI) from the live/dead cell staining kit (BioVision, Inc.). Poorly functioning or dead cells will be stained red, while live cells will be stained green.

4、对步骤3中细胞，用活死细胞染色试剂盒(BioVision,Inc)中1mM活染料和2.5mg/ml碘化丙啶(PI)进行染色，状态差或死的细胞会染红，活的细胞会变绿。

Fluorescence microscopy (excitation/emission, 488/518 nm) was used to detect stained live cells; (excitation/emission, 488/615 nm) was used to detect stained dead cells.

荧光显微镜(激发/发射，488/518nm)检测染色活细胞；(激发/发射，488/615)检测染色死亡细胞。

Three levels were randomly selected to count the number of surviving cells.

随机选择三个级别来计数存活细胞的数量。

[0104]

5. For the cells from step 3, add 100 µl of a mixture of CCK-8 and culture medium containing 10% CCK-8 reaction solution, incubate at 37°C for 2 h, and measure the optical density (OD) value at a wavelength of 450 nm. The control group was treated in the same way without the presence of hydrogel. Repeat the experiment for 3 groups.

5、对步骤3中细胞，加入含10%CCK-8反应液的CCK-8和培养基混合液100µl，37°C下孵育2h，测定450nm波长处的光密度(OD)值，对照组在没有水凝胶存在的情况下以同样的方式处理，重复3组实验。

The result is shown in Figure 4.

结果如图4。

[0105]

Example 8: Angiogenic capacity of naturally tissue-derived femoral periosteum ECM gel

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实施例8天然组织来源的股骨骨膜ECM凝胶的成血管能力

[0106]

1. The decellularization and gelation processes are the same as steps 1-11 in Example 1 above;

1、脱细胞及凝胶化处理步骤同上述实施例1中步骤1-11；

[0107]

2. Human umbilical vein endothelial cells were digested and resuspended to a cell density of 1×10 cells/mL. 100 μl of matrix gel containing 100 ng/ml VEGF, 8 mg/ml type I collagen, and 8 mg/ml decellularized periosteal ECM gel was added to each well of a 96-well plate and gelled at 37°C.

2、将人脐静脉内皮细胞消化重悬至1×10⁵个/mL细胞密度，在96孔板中加入分别含有100ng/ml VEGF，8mg/ml I型胶原和8mg/ml脱细胞骨膜ECM凝胶的基质凝胶，每孔100μl，37°C凝胶化。

[0108]

3. Add 100 μl of cell suspension to the hydrogel, incubate for 6 h, and then fix with 4% paraformaldehyde.

3、在水凝胶中加入100μl细胞悬液，培养6h后，用4%多聚甲醛固定。

[0109]

4. Quantify tubular structures by counting the number of intersections between branches of the endothelial cell network.

4、通过计数内皮细胞网络分支间的交叉数来定量管状结构。

[0110]

5. Five high-power fields under each condition were tested, and the experiment was repeated in three independent cultures.

5、对每个条件下的5个高功率场进行了检测，并在三种独立培养物中重复实验。

The results are shown in Figure 5.

结果如图5所示。

[0111]

Example 9: In vitro spontaneous mineralization evaluation of femoral periosteum ECM gel derived from natural tissues

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实施例9天然组织来源的股骨骨膜ECM凝胶的体外自发矿化评价

[0112]

1. The decellularization and gelation processes are the same as steps 1-11 in Example 1 above;

1、脱细胞及凝胶化处理步骤同上述实施例1中步骤1-11；

[0113]

2. Cure the material obtained in step 11 at 37°C.

2、将步骤11所得的材料37°C固化。

[0114]

3. The cured periosteal ECM gel was immersed in a simulated human body fluid m-SBF solution containing 100 μg/ml of polyaspartic acid (p-Asp) (1.67×10⁻³m CaCl2, 9.5×10⁻³m Na2HPO4, 150×10⁻³m NaCl), kept at a constant temperature of 4°C, and shaken for 28 days, with the solution being changed every 48 hours.

3、将固化骨膜ECM凝胶浸泡在含聚天冬氨酸(p-Asp)100μg/ml的模拟人体液m-SBF(1.67 ×10⁻³m CaCl2,9.5×10⁻³m Na2HPO4,150×10⁻³m NaCl)溶液中，恒温4°C，震荡28天，每48小时更换溶液。

[0115]

4. Rinse all mineralized collagen gels with deionized water after the specified time has elapsed.

4、所有矿化胶原凝胶在达到指定时间后，用去离子水冲洗。

[0116]

5. The mineralization of the samples was evaluated using micro-CT scanning, scanning electron microscopy, transmission electron microscopy, and von Kossa staining.

5、采用micro-CT扫描、扫描电镜、透射电镜和von Kossa染色法对样品进行矿化评价。

The results are shown in Figure 6.

结果如图6所示。

[0117]

Example 10 Osteogenic activity of femoral periosteum ECM gel derived from natural tissue

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实施例10天然组织来源的股骨骨膜ECM凝胶的成骨活性

[0118]

1. The decellularization and gelation processes are the same as steps 1-11 in Example 1 above;

1、脱细胞及凝胶化处理步骤同上述实施例1中步骤1-11；

[0119]

2. Primary osteoblasts of the skull were seeded in 24-well plates at a density of 1×10⁵/well.

2、将颅骨的原代成骨细胞以1×10⁵/孔的密度接种于24孔板内。

[0120]

3. Type I collagen hydrogel (8 mg/ml), periosteal ECM gel (4 mg/ml), periosteal ECM gel (8 mg/ml), and hydrogel-free incubation groups were placed in 24-well plates and cured at 37°C.

3、Ⅰ型胶原水凝胶8mg/ml，骨膜ECM凝胶4mg/ml，骨膜ECM凝胶8mg/ml，无水凝胶孵育等组分别置于24孔板内37°C固化。

[0121]

4. Add osteogenic induction medium (OIC, 100 nM dexamethasone, 5 μM ascorbic acid, 1 mM β-glycerophosphate) and incubate together, changing the medium every other day.

4、加入成骨诱导培养基(OIC,100nM地塞米松,5μM抗坏血酸,1mM b-甘油磷酸脂)共同孵育，每隔一天换一次液。

[0122]

5. At 14 and 28 days, gently wash the cells twice with PBS, fix with 4% paraformaldehyde for 15 minutes, then treat with 0.5% v/v Triton X-100 for 10 minutes, block with 10% goat serum, and then incubate overnight at 4°C with anti-Runx 2 antibody and anti-OCN antibody.

5、在14和28天，用PBS轻轻清洗细胞两次，用4%多聚甲醛固定15分钟，然后用0.5%v/v Triton x-100处理10分钟，用10%山羊血清封闭，然后4°C条件下敷：抗Runx 2抗体、抗OCN抗体过夜。

[0123]

6. Wash the cells with 1 ml of PBS, repeating the washing process three times.

6、用1ml PBS洗涤细胞，洗涤3次。

[0124]

7. Incubate the adapted secondary antibody at 37°C for 1 hour.

7、37°C条件下，孵育适应的二抗1h。

Stain with Rhodamine Phalloidin for 1 hour in the dark.

避光Rhodamine Phalloidin染色1h。

[0125]

8. Wash the cells with 1 ml of PBS, repeating the washing process three times.

8、用1ml PBS洗涤细胞，洗涤3次。

[0126]

9. After staining with DAPI for 10 min, wash the cells with 1 ml of PBS three times.

9、DAPI染色10min后，1ml PBS洗涤细胞，洗涤3次。

[0127]

10. Observed using an immunofluorescence microscope (Nikon Corporation, Minato, Tokyo, Japan).

10、通过免疫荧光显微镜(Nikon Corporation，Minato，日本东京)观察。

The result is shown in Figure 7.

结果如图7。

[0128]

Example 11: Effect of naturally derived femoral periosteum ECM gel on the repair of skull defects in rats.

实施例11天然组织来源的股骨骨膜ECM凝胶的大鼠颅骨缺损修复效果

[0129]

1. The decellularization and gelation processes are the same as steps 1-11 in Example 1 above;

1、脱细胞及凝胶化处理步骤同上述实施例1中步骤1-11；

[0130]

2. Eighteen SD rats aged 6-8 weeks (250±50g) were randomly divided into three groups (control group, type I collagen hydrogel group, and periosteal hydrogel group).

2、取18只6-8周龄的SD大鼠(250±50g)随机分为3组(对照组、Ⅰ型胶原水凝胶组、骨膜水凝胶组)。

[0131]

3. Anesthetize animals by intraperitoneal injection of sodium pentobarbital (50 mg/kg).

3、腹腔注射戊巴比妥钠(50mg/kg)麻醉动物。

After disinfection, a skull defect model was created. The diameter of the bilateral full-thickness critical-size skull defect was 4 mm (skull thickness ≈ 0.5 mm, volume ≈ 6.28 mm³).

消毒后行颅骨缺损造模，双侧全厚度临界大小颅骨缺损直径为4mm(颅骨厚度≈0.5mm，体积≈6.28mm³)。

[0132]

4. The control group was not filled, but the periosteal defects were filled with 8mg/ml type I collagen hydrogel and 8mg/ml periosteal hydrogel.

4、对照组不填充，将8mg/ml I型胶原水凝胶、8mg/ml骨膜水凝胶填充骨膜缺损部位。

Then, the periosteum and scalp were sutured together with intermittent 4-0 nylon sutures.

然后，用间断的4-0尼龙缝线缝合骨膜和头皮。

[0133]

5. Four and eight weeks after transplantation, animals were euthanized by CO_NER13 and the skull was fixed in 4% paraformaldehyde.

5、移植后4、8周，用CO₂窒息处死动物，将头骨固定在4%多聚甲醛内。

[0134]

6. The repair effect of the material was analyzed using micro-CT (Siemens Ineon, German Esborne) based on the scanning method (voltage 80KV, current 500mA, isotropic resolution 14.97mm).

6、根据扫描方式(电压为80KV，电流为500mA，各向同性分辨率为14.97mm)，采用micro-ct(西门子Ineon，德国埃斯伯恩)对材料的修复效果进行了分析。

New bone formation was analyzed using Ineon ResearchWorkine 2.2 (Siemens).

采用IneonResearchWorkine2.2(西门子)对新骨形成进行了分析。

The result is shown in Figure 8.

结果如图8。

[0135]

Example 12: In vivo osteogenic repair effect of femoral periosteum ECM gel derived from natural tissue

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实施例12天然组织来源的股骨骨膜ECM凝胶的在体成骨修复效果

[0136]

1. The decellularization and gelation processes are the same as steps 1-11 in Example 1 above;

1、脱细胞及凝胶化处理步骤同上述实施例1中步骤1-11；

[0137]

2. 1 ml of type I collagen and femoral periosteum ECM gel were subcutaneously injected into the back of SD rats through a 26 mm needle.

2、将1ml Ⅰ型胶原、股骨骨膜ECM凝胶分别经26mm针皮下注射至SD大鼠背部。

[0138]

3. Skin tissue samples were collected on days 3, 7, 21, and 28, and the obtained tissues were fixed in 4% (w/v) paraformaldehyde (PFA) for 24 hours.

3、分别于第3、7、21、28天采集皮肤组织标本，将获得的组织固定在4%(w/v)多聚甲醛(PFA)中24小时。

[0139]

4. After washing with PBS for 5 min × 3 times, dehydrate with 50, 75, 85, 95 and 100% ethanol at consecutive concentrations, and embed in paraffin.

4、PBS洗涤5min×3次后，用50、75、85、95、100%乙醇连续浓度脱水，石蜡包埋。

[0140]

5. The paraffin sections were then cut into 7μm thick pieces, and the obtained tissues were analyzed by staining with hematoxylin and eosin (HE).

5、然后将石蜡切片切成7μm厚，通过苏木精和伊红(HE)染色对获得组织进行分析。

[0141]

6. Take rat heart, liver, spleen, lung and kidney for visceral toxicity assessment.

6、取大鼠心脏、肝、脾、肺、肾进行内脏毒性评估。

The result is shown in Figure 9.

结果如图9。

[0142]

The natural tissue-derived periosteal ECM gel materials obtained in Examples 2-4 were subjected to histological evaluation, surface property observation, biocompatibility analysis,

and in vivo repair experiments. The results were similar to those of the porcine femoral periosteal ECM gel materials in Examples 5-12. This indicates that by adjusting the reagent concentration and treatment time determined after the above optimization, it is possible to prepare natural tissue-derived periosteal ECM gel materials with similar effects. These materials have good repair and regeneration effects and can be used as filling materials for clinical treatment of bone defects.

对实施例2-4所得的天然组织来源的骨膜ECM凝胶材料分别进行组织学评价、表面性质观察及生物相容性分析以及材料的在体修复实验评价，结果与实施例5-12中猪股骨骨膜ECM凝胶材料结果类似，这表明可通过上述优化后确定的试剂浓度和处理时间的调整，实现效果类似的天然组织来源的骨膜ECM凝胶材料的制备，其具有良好的修复再生作用，可以作为临床上治疗骨缺损填充材料。

[0143]

As can be seen from the above embodiments, the method for preparing the periosteum ECM gel material from natural tissue sources provided by the present invention can induce biological mineralization, has osteogenic induction activity, is beneficial to osteogenic repair in vivo, and can also promote endothelial cell differentiation and promote angiogenesis in vivo.

由上述实施例可知，本发明提供的天然组织来源的骨膜ECM凝胶材料制备方法，该材料可以诱发生物矿化，具有成骨诱导活性，有利于体内成骨修复，还可以促进内皮细胞分化，促进体内血管生成。

Specific functions can be combined through cross-linking agent modification and the addition of growth factors.

经交联剂修饰和生长因子添加可组合特定功能。

It is a promising material for bone defect transplantation.

是一种很有潜力的骨缺损移植材料。

[0144]

The above description is only a preferred embodiment of the present invention. It should be noted that for those skilled in the art, several improvements and modifications can be made without departing from the principle of the present invention, and these improvements and modifications should also be considered within the scope of protection of the present invention.

以上所述仅是本发明的优选实施方式，应当指出，对于本技术领域的普通技术人员来说，在不脱离本发明原理的前提下，还可以做出若干改进和润饰，这些改进和润饰也应视为本发明的保护范围。