Notice

DESCRIPTION WO2023040853A1

Reconstruction of the soft tissue-bone immune repair environment: periosteum-bone complex and its preparation method Technical Field

---

重建软组织-骨免疫修复环境骨膜-骨复合体及制备方法 技术领域

[0001]

This invention mainly relates to the biological field for bone tissue regeneration and repair, specifically a periosteum-bone complex and its preparation method aimed at reconstructing

the soft tissue-bone immune repair environment. This material focuses on soft tissue damage accompanying bone defects and its impact on bone repair, and rationally utilizes the advantages of spatial composite scaffolds to promote bone regeneration and repair.

本发明主要涉及用于骨组织再生修复的生物领域，具体是一种旨在重建软组织-骨免疫修复环境的骨膜-骨复合体及其制备方法，该材料关注骨缺损伴随的软组织损伤及其对骨修复的影响，合理运用空间复合模式支架的优势促进骨再生修复。

Background Technology

背景技术

[0002]

Bone defects caused by trauma, tumors, and infections are common clinical conditions that affect human health. In particular, multiple and complex bone defects often require repeated treatments, causing heavy psychological stress and economic burden on individuals and potentially impacting social stability. This is a pressing problem that clinical orthopedics needs to solve.

由创伤、肿瘤和感染等原因引起的骨缺损是临床常见病，影响人类的健康，特别是多发的复杂性骨缺损，往往需要反复治疗，对个人造成沉重的心理压力及经济负担，对社会稳定产生潜在的影响，是临床骨科亟待解决的难题。

[0003]

In recent years, biomaterial replacement therapy has gradually gained attention. Among them, high-stiffness single-phase bone substitutes (such as bioceramics and bioglass) have been widely used due to their similarity to the mineral composition of bone.

近年，生物材料的替代治疗逐渐受到人们重视，其中，高刚度的单相骨替代品(如生物陶瓷、生物玻璃)由于与骨的矿物组成相似而得到广泛应用。

Bone healing is a complex physiological process that includes early inflammation regulation, angiogenesis, osteogenic differentiation, and biomineralization. These alternative materials undoubtedly contribute to bone formation, but their advantages in immune regulation and angiogenesis are limited. The regulation of the local immune microenvironment can be improved by adding chemical molecules and increasing surface coatings, but these artificial synthesis or local addition methods are still very different from physiological conditions, and there are difficulties in controlling the amount of chemical molecules used and the release process. In addition, severe bone defects are often accompanied by damage to adjacent soft tissues. Severe muscle trauma can exacerbate the recruitment of macrophages to the site of

injury, thereby impairing bone healing. Therefore, seeking more suitable alternatives for bone repair remains of great importance.

骨愈合作为一个复杂的生理过程，包括了早期炎症调节、血管生成、成骨分化和生物矿化，这些替代材料无疑有助于成骨，但在免疫调节、血管生成方面的优势有限。 通过加入化学分子、增加表面涂层等方式可以改善对局部免疫微环境的调节，但这些人工合成或局部添加的方式与生理条件仍有很大差异，而且在化学分子的用量、释放过程等多环节中存在控制难点。 另外，严重的骨缺损通常也会伴随相邻软组织的损害，严重的肌肉创伤会加剧巨噬细胞向损伤部位的募集，从而损害骨愈合。 因此，寻求更加合理的骨修复替代品仍然有重要意义。

[0004]

Natural bone is a highly vascularized hard tissue covered by a soft periosteum. The periosteum can act as a cellular barrier against excessive infiltration of inflammatory cells in the trauma environment, and also as a physical barrier to prevent muscle, fibrous tissue, and other tissues from filling the defect.

天然骨是由软质的骨膜覆盖的高度血管化的硬质组织，骨膜可以作为抵抗创伤环境中炎性细胞过度渗透的细胞屏障，也是阻隔肌肉、纤维组织等填塞缺损部位的物理屏障。

Moreover, the periosteum provides the cortical bone with ample blood supply and nutrition, and also provides a supportive microenvironment for the differentiation and maturation of

mesenchymal stem cells. In addition, reports indicate that hydrogels derived from periosteal extracellular matrix can enhance M2 polarization of macrophages in the early stages of bone injury, thereby exerting an immunomodulatory function.

而且，骨膜为皮质骨提供了充足的血供和营养，也为间充质干细胞分化成熟提供了支持性的微环境。 另外，报道显示，骨膜细胞外基质来源的水凝胶可在骨损伤早期增强巨噬细胞的M2极化，发挥免疫调节功能。

[0005]

Given the natural advantages of the periosteum in terms of physical barrier, immune regulation and angiogenesis, combined with the osteogenic properties of cortical bone itself, this invention uses an innovative extracellular matrix preparation technology to obtain a spatially patterned periosteum-bone complex, demonstrating its ability to regulate the early immune microenvironment of soft tissue-bone injury and coordinate subsequent angiogenesis and osteogenic events to promote bone healing.

鉴于骨膜在物理阻隔、免疫调节和血管生成等方面的天然优势，结合皮质骨本身的成骨性能，本发明通过创新的细胞外基质制备技术来获得空间模式的骨膜-骨复合体，证明其具备调节软组织-骨损伤早期免疫微环境的性能，并协调后续成血管、成骨事件，促进骨愈合。

Currently, there are no reports on the preparation of periosteal-bone complexes.

目前，尚无有关骨膜-骨复合体制备的报道。

[0006]

Summary of the Invention

发明内容

[0007]

The purpose of this invention is to fill the gap in the existing technology where single-phase bone repair biological scaffolds are difficult to effectively avoid inflammatory infiltration from soft tissue, which aggravates insufficient immune regulation in the early stage of bone defect repair. Therefore, this invention provides a spatial model periosteum-bone complex and its preparation method, which aims to reconstruct the soft tissue-bone immune repair environment.

本发明目的在于填补现有技术中，单相骨修复生物支架难以有效避免软组织来源炎症浸润，加重骨缺损修复早期免疫调节不足，因此提供一种旨在重建软组织-骨免疫修复环境的空间模式骨膜-骨复合体及其制备方法。

[0008]

To achieve the above objectives, the present invention employs the following technical solution: a method for preparing a spatially patterned periosteum-bone complex from natural animal sources, comprising the following steps:

为了实现上述目的，本发明采用如下技术方案，天然动物来源的空间模式骨膜-骨复合体的制备方法，包括以下步骤：

[0009]

(1) Take the femur of an adult large white pig slaughtered within 12 hours, separate the metaphysis, collect the femur shaft, remove the internal medullary cavity, cut and sample the femur, and divide it into 10mm*8mm pieces.

---

(1)取宰杀12h以内的成年大白猪的股骨，分离干骺端，收集股骨干部分，去除内部骨髓腔，将股骨切割打样，分割成10mm*8mm片状；

[0010]

(2) Take a certain amount of bone slices, place them on a low temperature (4°C) shaker at 100 rpm, and rinse them three times with PBS buffer containing protease inhibitors for 10 min each time to remove blood, attached fat fragments and other impurities.

---

(2)取一定量的片状骨，置于低温(4°C)摇床100rpm震荡，用含蛋白酶抑制剂的PBS缓冲液漂洗3次，每次10min，去除血液、贴附的脂肪碎片和其他杂质；

[0011]

(3) The pretreated bone sheets were placed in an EDTA-Na2 solution and ultrasonically decalcified for 12 days.

---

(3)将上述预处理的片状骨置于乙二胺四乙基二钠(EDTA-Na2)溶液中超声脱钙12天；

[0012]

(4) The decalcified bone sheets were subjected to three cycles of freeze-thaw in liquid nitrogen (-80°C/22°C);

---

(4)将脱钙处理后的片状骨用液氮冻融3个循环(-80°C/22°C)；

[0013]

(5) In PBS buffer containing Triton X, shake at 100 rpm for 12-36 hours at low temperature (4° C);

---

(5)在含Triton X的PBS缓冲液中，低温(4°C)摇床100rpm震荡12-36小时；

[0014]

(6) In PBS buffer containing sodium dodecyl ether sulfate (SLES), shake at 100 rpm for 1-4 hours at low temperature (4°C);

---

(6)在含十二烷基醚硫酸钠磺酸(SLES)的PBS缓冲液中，低温(4°C)摇床100rpm震荡1-4小时；

[0015]

(7) Wrap the periosteal portion of the periosteum-bone complex with sealing film to minimize its external exposure;

---

(7)用封口膜具包裹骨膜-骨复合体的骨膜部分，使其尽可能不对外暴露；

[0016]

(8) Place the mixture in PBS buffer containing SLES and shake at 100 rpm for 12-36 hours at low temperature (4°C).

---

(8)再次在含SLES的PBS缓冲液中，低温(4°C)摇床100rpm震荡12-36小时；

[0017]

(9) In PBS buffer containing DNase I enzyme, shake at 37°C and 100 rpm for 12-24 hours;

---

(9)在含DNase I酶的PBS缓冲液中，37°C摇床100rpm震荡12-24小时；

[0018]

(10) In Tris-HCl buffer, shake at 100 rpm for 6-24 hours at low temperature (4°C);

---

(10)在三羟甲基氨基甲烷盐酸盐(Tris-HCl)缓冲液中，低温(4°C)摇床100rpm震荡6-24小时；

[0019]

(11) Add the mixed antibacterial solution to sterile physiological saline and shake at 100 rpm for 6-24 hours at low temperature (4°C);

---

(11)在无菌生理盐水中，加入混合抗菌溶液，低温(4°C)摇床100rpm震荡6-24小时；

[0020]

(12) Repeat steps (10) and (11) above three times to obtain periosteal-bone composite material derived from natural tissue.

---

(12)上述(10)、(11)步骤重复3次，得到天然组织来源的骨膜-骨复合体材料。

[0021]

In steps (5)-(9), rinse with double-distilled water for 3-6 hours after each step is completed.

步骤(5)-(9)中，每个步骤完成后均用双蒸水冲洗3-6小时。

[0022]

In addition, the decalcification solution containing EDTA-Na₂ has an EDTA-Na₂ mass concentration of 5%-20%;

另外，含EDTA-Na₂的脱钙液，EDTA-Na₂质量浓度为5%-20%；

[0023]

PBS buffer containing protease inhibitors, with a protease inhibitor concentration of 10-50 K IU/ml;

含蛋白酶抑制剂的PBS缓冲液，蛋白酶抑制剂浓度为10-50K IU/ml；

[0024]

The PBS buffer containing Triton X is Triton X-100 PBS buffer with a mass concentration of 0.01%-5%;

含Triton X的PBS缓冲液为质量浓度0.01%-5%的Triton X-100 PBS缓冲液；

[0025]

SLES-containing PBS buffer is a PBS buffer with a mass concentration of 0.01%-5% SLES;

含SLES的PBS缓冲液为质量浓度0.01%-5%的SLES的PBS缓冲液；

[0026]

The PBS buffer containing DNase I is a PBS buffer with a concentration of 1-2 mg/mL;

含DNase Ⅰ的PBS缓冲液为浓度为1-2mg/mL的PBS缓冲液；

[0027]

Buffer containing Tris-HCl, with a Tris-HCl concentration of 5-20 mM;

含Tris-HCl缓冲液,Tris-HCl浓度为5-20mM；

[0028]

The concentrations of penicillin and streptomycin in the mixed antibacterial solution were 20 U/ml and 20 μg/ml, respectively; the ratio of penicillin to streptomycin was 1:1; and the volume ratio of the added mixed antibacterial solution to the original solution was 5:1.

混合抗菌液中青霉素和链霉素的浓度分别为20U/ml，20μg/ml；青霉素和链霉素的比例为1:1，加入的混合抗菌液与原溶液体积比为5:1。

[0029]

This invention addresses the problems existing in current clinical soft tissue replacement products for bone injuries by establishing a method for preparing a periosteal-bone complex scaffold derived from natural allogeneic tissue. The scaffold is obtained by cutting and sampling the femoral shaft of an adult Large White pig, repeatedly rinsing and freezing and

thawing, and detoxifying it with PBS buffer containing protease inhibitors, PBS buffer containing Triton X-100, PBS buffer containing SLES, PBS buffer containing DNase I, and Tris-HCl buffer.

本发明针对目前用于临床软组织合并骨损伤替代品存在的问题，建立了一种天然异体组织来源的骨膜-骨复合体支架的制备方法，该支架由成年大白猪股骨干切割打样、反复漂洗冻融、含蛋白酶抑制剂的PBS缓冲液、含Triton X-100的PBS缓冲液、含SLES的PBS缓冲液、含DNase Ⅰ的PBS缓冲液和Tris-HCl缓冲液脱毒处理后获得。

As can be seen from the above preparation method, compared with the prior art, the present invention has the following main advantages:

经由上述的制备方案可知，与现有技术相比，本发明公开具有以下主要优点：

[0030]

(1) The periosteum-bone scaffold used in this invention is of allogeneic natural origin and is a spatial pattern periosteum-bone complex obtained by an innovative method combining physical freeze-thaw, ultrasonic decalcification and combined decellularization techniques.

---

(1)本发明所采用的骨膜-骨支架是异体天然来源的，运用物理冻融、超声脱钙、联合脱细胞技术相结合的创新方法获得的空间模式的骨膜-骨复合体。

[0031]

(2) The material of the present invention is detoxified by repeated rinsing with Tris-HCl buffer and sterile saline. The scaffold has low cytotoxicity, good biocompatibility and main bioactive components, and has a complex natural structure that is difficult to replicate.

---

(2)本发明材料通过Tris-HCl缓冲液及无菌生理盐水反复漂洗进行脱毒，支架细胞毒性低，拥有良好的生物相容性和主要的生物活性成分，并具有难以复制的天然复杂结构。

[0032]

(3) The material prepared by the present invention can act as an immune barrier in the early stage of bone repair and participate in macrophage phenotypic transformation.

---

(3)本发明所制备的材料在骨修复早期可作为免疫屏障参与巨噬细胞表型转化。

[0033]

(4) The material prepared by the present invention has the ability to coordinate early immune regulation and subsequent osteogenic-angiogenic events and promote bone repair.

---

(4)本发明所制备的材料具有协调早期免疫调节和后续成骨-血管生成事件，具备促进骨修复的性能。

Attached Figure Description

附图说明

[0034]

Figure 1 is a general diagram and CT scan of the material of the present invention;

图1是本发明材料大体图及CT扫描图；

[0035]

Figure 2 shows the H&E staining, DAPI staining, and DNA quantification detection results of the materials of this invention.

图2是本发明材料H&E染色、DAPI染色图和DNA定量检测图；

[0036]

Figure 3 shows the Sirius red staining, polarized light observation, and quantitative detection of collagen and GAGs in the material of this invention.

图3是本发明材料天狼星红染色、偏正光观察，胶原和GAGs定量检测图；

[0037]

Figure 4 is a scanning electron microscope image of the microstructure of various parts of the material of the present invention;

图4是本发明材料各个部分微观结构扫描电子显微镜图；

[0038]

Figure 5 shows the atomic force microscope image, pressure-strain curve, and stiffness calculation value of the material of the present invention.

图5是本发明材料原子力显微镜图、压力-应变曲线图和刚度计算值；

[0039]

Figure 6 shows the inhibition of macrophage M1 polarization and the inhibition of inflammation-related gene expression by the material of the present invention;

图6是本发明材料抑制巨噬细胞M1极化、抑制炎性相关基因表达图；

[0040]

Figure 7 shows how the material of this invention promotes M2 polarization in macrophages and the expression of anti-inflammatory related genes.

图7是本发明材料促进巨噬细胞M2极化、促进抗炎性相关基因表达图；

[0041]

Figure 8 shows the expression of osteogenic-related markers promoted by the material of the present invention;

图8是本发明材料促进成骨相关标志物的表达；

[0042]

Figure 9 shows the effect of the material of the present invention on blood vessel migration;

图9是本发明材料促进血管迁入图；

[0043]

Figure 10 shows a CT scan and histological evaluation of the material of the present invention promoting bone repair.

图10是本发明材料促进骨修复的CT扫描图和组织学评估图。

Detailed Implementation

具体实施方式

[0044]

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

下面结合附图及实施示例对本发明进行完整详细描述，但本发明的实施不仅限于此。

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

[0045]

Example 1: Periosteum-bone complex designed to reconstruct the immune repair environment of soft tissue and bone and its preparation method

---

实施例1：旨在重建软组织-骨免疫修复环境的骨膜-骨复合体及其制备方法

[0046]

(1)Acquisition of materials

---

(1)取材

[0047]

Take the femur from a healthy adult Large White pig slaughtered within 12 hours, separate the metaphysis, collect the femoral shaft, remove the internal medullary cavity, and cut the femoral shaft into 10mm*8mm pieces.

取宰杀12h以内健康成年大白猪的股骨，分离干骺端，收集股骨干部分，去除内部骨髓腔，将股骨干分割成10mm*8mm片状；

[0048]

(2) Pretreatment

---

(2)预处理

[0049]

Take a certain amount of bone slices, place them on a low-temperature (4°C) shaker at 100 rpm, and rinse them three times with PBS buffer containing protease inhibitors for 10 min each time to remove blood, attached fat fragments and other impurities.

取一定量的片状骨，置于低温(4°C)摇床100rpm震荡，用含蛋白酶抑制剂的PBS缓冲液漂洗3次，每次10min，去除血液、贴附的脂肪碎片和其他杂质；

[0050]

(3) Decalcification

---

(3)脱钙

[0051]

The pretreated bone sheets were placed in a 20% (w/v) EDTA-2Na solution and ultrasonically decalcified for 12 days. The ultrasonic parameters were 40 kHz and the temperature was 22 °C.

将上述预处理的片状骨置于20%(w/v)EDTA-2Na溶液中超声脱钙12天，超声参数为40kHZ，温度22°C；

[0052]

(4) Obtaining the periosteal-bone complex

---

(4)骨膜-骨复合体获取

[0053]

① Three cycles of liquid nitrogen freeze-thaw (-80°C/37°C);

---

①液氮冻融3个循环(-80°C/37°C)；

[0054]

② Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to PBS buffer containing 1% Triton X-100, and shake at 100 rpm for 12 hours at low temperature (4°C);

---

②在浓度为1%的Triton X-100的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡12小时；

[0055]

③ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1% deionized water containing SLES, and shake at 100rpm for 4 hours at low temperature (4°C);

---

③在浓度为1%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡4小时；

[0056]

④ Wrap the periosteum portion of the periosteum-bone complex with sealing film to minimize its external exposure;

---

④用封口膜具包裹骨膜-骨复合体的骨膜部分，使其尽可能不对外暴露；

[0057]

⑤ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1% SLES-containing deionized water, and shake at 100rpm for 12 hours at low temperature (4°C).

---

⑤在浓度为1%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡12小时；

[0058]

⑥ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1mg/ml PBS buffer containing DNase I, and shake at 100rpm for 12 hours at 37°C to obtain the periosteal bone scaffold.

---

⑥在浓度为1mg/ml的含DNase I的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，37°C摇床100rpm震荡12小时，即可得到骨膜骨支架。

[0059]

(5) Detoxification of the periosteal-bone complex

---

(5)骨膜-骨复合体脱毒

[0060]

① In Tris-HCl buffer, shake at 100 rpm on a low temperature (4°C) for 24 hours;

---

①在三羟甲基氨基甲烷盐酸盐(Tris-HCl)缓冲液中，低温(4°C)摇床100rpm震荡24小时；

[0061]

② Add the mixed antibacterial solution to sterile physiological saline and shake at 100 rpm for 24 hours at low temperature (4°C);

---

②在无菌生理盐水中，加入混合抗菌溶液，低温(4°C)摇床100rpm震荡24小时；

[0062]

③ Repeat steps ① and ② above three times.

---

③上述①、②两个步骤重复三次。

[0063]

After processing through the steps in Example 1, a periosteal-bone complex with no cytotoxicity, low immunogenicity, good tissue activity, and a spatial composite structure was finally obtained.

经过实例1中各个步骤处理，最终获得无细胞毒性、低免疫原性、良好组织活性成分和空间复合结构的骨膜-骨复合体。

[0064]

1.

1.

In Example 1, the gross view and decellularization assessment of the periosteum-bone complex scaffold.

实例1中，骨膜-骨复合体支架的大体观及脱细胞评估

[0065]

The gross appearance and CT scan of the periosteal-bone complex scaffold are shown in Figure 1.

骨膜-骨复合体支架的大体外观图及CT扫描图，如图1。

[0066]

2.

2.

In Example 1, the decellularization assessment of the periosteum-bone complex scaffold.

实例1中，骨膜-骨复合体支架的脱细胞评估

[0067]

H&E staining showed no cells or residual nuclear components, and DNA quantification showed almost no DNA components, as shown in Figure 2.

H&E染色显示无细胞及无细胞核成分残留，DNA定量检测几乎不含有DNA成分，如图2。

[0068]

3.

3.

Microstructural evaluation of the periosteum-bone complex scaffold in Example 1

实例1中，骨膜-骨复合体支架的微观结构评估

[0069]

The collagen arrangement, bone crystal structure, and periosteum-bone interface connection structure of the present invention were observed using a scanning electron microscope, as shown in Figure 3.

扫描电子显微镜观察本发明的胶原排布、骨结晶结构以及骨膜-骨交界面连接结构，如图3。

[0070]

4.

4.

In Example 1, the surface morphology and mechanical evaluation of the periosteum-bone complex scaffold.

实例1中，骨膜-骨复合体支架的表面形貌及机械评估

[0071]

The microscopic surface morphology of the present invention was observed using an atomic force microscope, as shown in Figure 4.

原子力显微镜观察本发明微观表面形貌，如图4。

[0072]

Mechanical testing and evaluation records the pressure-strain curves, and the stiffness of the periosteum and bone phases in the spatial model periosteum-bone framework are calculated, as shown in Figure 5.

机械力学测试评估记录压力-应变曲线，计算空间模式骨膜骨支架骨膜相和骨相的刚度，如图5。

[0073]

5.

5.

In Example 1, the immunomodulatory function of the periosteum-bone complex scaffold was verified.

实例1中，骨膜-骨复合体支架的免疫调节功能验证

[0074]

The periosteal-bone complex scaffold influences macrophage phenotype. The periosteal phase inhibits macrophage polarization toward the M1 direction and suppresses the expression of inflammation-related genes; the bone phase inevitably induces macrophage polarization toward the M1 direction, as shown in Figure 6.

骨膜-骨复合体支架影响巨噬细胞表型，骨膜相抑制巨噬细胞向M1方向极化，抑制炎症相关基因表达；骨相无法避免地诱导巨噬细胞向M1方向极化，如图6；

[0075]

The periosteal phase of the periosteum-bone complex scaffold induces macrophage polarization toward the M2 direction, promoting the expression of anti-inflammatory related genes, as shown in Figure 7.

骨膜-骨复合体支架骨膜相诱导巨噬细胞向M2方向极化，促进抗炎性相关基因表达，如图7。

[0076]

6.

6.

In Example 1, the osteogenic properties of the periosteum-bone complex scaffold were verified.

实例1中，骨膜-骨复合体支架促进成骨性能验证

[0077]

The periosteal-bone complex scaffold promotes the expression of osteogenic-related genes (Runx2, ALP, Col 1a1, OPN, OCN), as shown in Figure 8.

骨膜-骨复合体支架促进成骨相关基因(Runx2,ALP,Col 1a1,OPN,OCN)表达，如图8。

[0078]

7.

7.

In Example 1, the angiogenesis-promoting performance of the periosteal-bone complex scaffold was verified.

实例1中，骨膜-骨复合体支架促进成血管性能验证

[0079]

The periosteal-bone complex scaffold promotes angiogenesis and migration, as shown in Figure 9.

骨膜-骨复合体支架促进血管形成及迁入，如图9。

[0080]

8.

8.

In Example 1, the assessment of the periosteal-bone complex scaffold's ability to promote bone repair.

实例1中，骨膜-骨复合体支架促进骨修复的评估

[0081]

Rats with bone defects were divided into four groups: simple defect group, softened decellularized bone treatment group, and periosteal-bone complex treatment group.

将骨缺损大鼠分为四组，分别为单纯缺损组、软化的脱细胞骨治疗组、骨膜-骨复合体治疗组。

The corresponding scaffolds were filled into the bone defects respectively.

分别于骨缺损处填充相应支架。

After 4 and 8 weeks of treatment, it was found that the periosteal-bone complex treatment group had significantly better results than the other three groups.

治疗4周及8周后可发现，骨膜-骨复合体治疗组效果显著优于其余三组。

As shown in Figure 10.

如图10。

[0082]

Example 2: Periosteum-bone complex designed to reconstruct the immune repair environment of soft tissue and bone and its preparation method

---

实施例2：旨在重建软组织-骨免疫修复环境的骨膜-骨复合体及其制备方法

[0083]

(1)Acquisition of materials

---

(1)取材

[0084]

Take the femur from a healthy adult Large White pig slaughtered within 12 hours, separate the metaphysis, collect the femoral shaft, remove the internal medullary cavity, and cut the femoral shaft into 10mm*8mm pieces.

取宰杀12h以内健康成年大白猪的股骨，分离干骺端，收集股骨干部分，去除内部骨髓腔，将股骨干分割成10mm*8mm片状；

[0085]

(2) Pretreatment

---

(2)预处理

[0086]

Take a certain amount of bone slices, place them on a low-temperature (4°C) shaker at 100 rpm, and rinse them three times with PBS buffer containing protease inhibitors for 10 min each time to remove blood, attached fat fragments and other impurities.

取一定量的片状骨，置于低温(4°C)摇床100rpm震荡，用含蛋白酶抑制剂的PBS缓冲液漂洗3次，每次10min，去除血液、贴附的脂肪碎片和其他杂质；

[0087]

(3) Decalcification

---

(3)脱钙

[0088]

The pretreated bone sheets were placed in a 20% (w/v) EDTA-2Na solution and ultrasonically decalcified for 12 days. The ultrasonic parameters were 40 kHz and the temperature was 22 °C.

将上述预处理的片状骨置于20%(w/v)EDTA-2Na溶液中超声脱钙12天，超声参数为40kHZ，温度22°C；

[0089]

(4) Obtaining the periosteal-bone complex

---

(4)骨膜-骨复合体获取

[0090]

① Three cycles of liquid nitrogen freeze-thaw (-80°C/37°C);

---

①液氮冻融3个循环(-80°C/37°C)；

[0091]

② Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 2% Triton X-100 PBS buffer, and shake at 100rpm for 24 hours at low temperature (4°C).

---

②在浓度为2%的Triton X-100的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡24小时；

[0092]

③ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 2% SLES-containing deionized water, and shake at 100rpm for 4 hours at low temperature (4°C);

---

③在浓度为2%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡4小时；

[0093]

④ Wrap the periosteum portion of the periosteum-bone complex with sealing film to minimize its external exposure;

---

④用封口膜具包裹骨膜-骨复合体的骨膜部分，使其尽可能不对外暴露；

[0094]

⑤ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 2% SLES-containing deionized water, and shake at 100rpm for 12 hours at low temperature (4°C).

---

⑤在浓度为2%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡12小时；

[0095]

⑥ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1mg/ml PBS buffer containing DNase I, and shake at 100rpm for 12 hours at 37°C to obtain the periosteal bone scaffold.

---

⑥在浓度为1mg/ml的含DNase I的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，37°C摇床100rpm震荡12小时，即可得到骨膜骨支架。

[0096]

(5) Detoxification of the periosteal-bone complex

---

(5)骨膜-骨复合体脱毒

[0097]

① In Tris-HCl buffer, shake at 100 rpm on a low temperature (4°C) for 24 hours;

---

①在三羟甲基氨基甲烷盐酸盐(Tris-HCl)缓冲液中，低温(4°C)摇床100rpm震荡24小时；

[0098]

② Add the mixed antibacterial solution to sterile physiological saline and shake at 100 rpm for 24 hours at low temperature (4°C);

---

②在无菌生理盐水中，加入混合抗菌溶液，低温(4°C)摇床100rpm震荡24小时；

[0099]

③ Repeat steps ① and ② above three times.

---

③上述①、②两个步骤重复三次。

[0100]

Ultimately, a periosteum-bone complex with no cytotoxicity, low immunogenicity, good tissue activity, and a spatial composite structure was obtained.

最终获得无细胞毒性、低免疫原性、良好组织活性成分和空间复合结构的骨膜-骨复合体。

[0101]

Example 3: Periosteum-bone complex designed to reconstruct the immune repair environment of soft tissue and bone and its preparation method

---

实施例3：旨在重建软组织-骨免疫修复环境的骨膜-骨复合体及其制备方法

[0102]

(1)Acquisition of materials

---

(1)取材

[0103]

Take the femur from a healthy adult Large White pig slaughtered within 12 hours, separate the metaphysis, collect the femoral shaft, remove the internal medullary cavity, and cut the femoral shaft into 10mm*8mm pieces.

取宰杀12h以内健康成年大白猪的股骨，分离干骺端，收集股骨干部分，去除内部骨髓腔，将股骨干分割成10mm*8mm片状；

[0104]

(2) Pretreatment

---

(2)预处理

[0105]

Take a certain amount of bone slices, place them on a low-temperature (4°C) shaker at 100 rpm, and rinse them three times with PBS buffer containing protease inhibitors for 10 min each time to remove blood, attached fat fragments and other impurities.

取一定量的片状骨，置于低温(4°C)摇床100rpm震荡，用含蛋白酶抑制剂的PBS缓冲液漂洗3次，每次10min，去除血液、贴附的脂肪碎片和其他杂质；

[0106]

(3) Decalcification

---

(3)脱钙

[0107]

The pretreated bone sheets were placed in a 20% (w/v) EDTA-2Na solution and ultrasonically decalcified for 12 days. The ultrasonic parameters were 40 kHz and the temperature was 22 ° C.

将上述预处理的片状骨置于20%(w/v)EDTA-2Na溶液中超声脱钙12天，超声参数为40kHZ，温度22°C；

[0108]

(4) Obtaining the periosteal-bone complex

---

(4)骨膜-骨复合体获取

[0109]

① Three cycles of liquid nitrogen freeze-thaw (-80°C/37°C);

---

①液氮冻融3个循环(-80°C/37°C)；

[0110]

② Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to PBS buffer containing 1% Triton X-100, and shake at 100 rpm for 12 hours at low temperature (4°C);

---

②在浓度为1%的Triton X-100的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡12小时；

[0111]

③ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 0.5% SLES-containing deionized water, and shake at 100rpm for 4 hours at low temperature (4°C);

---

③在浓度为0.5%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡4小时；

[0112]

④ Wrap the periosteum portion of the periosteum-bone complex with sealing film to minimize its external exposure;

---

④用封口膜具包裹骨膜-骨复合体的骨膜部分，使其尽可能不对外暴露；

[0113]

⑤ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 0.5% SLES-containing deionized water and shake at 100 rpm for 24 hours at low temperature (4°C).

---

⑤在浓度为0.5%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡24小时；

[0114]

⑥ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1mg/ml PBS buffer containing DNase I, and shake at 100rpm for 12 hours at 37°C to obtain the periosteal bone scaffold.

---

⑥在浓度为1mg/ml的含DNase I的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，37°C摇床100rpm震荡12小时，即可得到骨膜骨支架。

[0115]

(5) Detoxification of the periosteal-bone complex

---

(5)骨膜-骨复合体脱毒

[0116]

① In Tris-HCl buffer, shake at 100 rpm on a low temperature (4°C) for 24 hours;

---

①在三羟甲基氨基甲烷盐酸盐(Tris-HCl)缓冲液中，低温(4°C)摇床100rpm震荡24小时；

[0117]

② Add the mixed antibacterial solution to sterile physiological saline and shake at 100 rpm for 24 hours at low temperature (4°C);

---

②在无菌生理盐水中，加入混合抗菌溶液，低温(4°C)摇床100rpm震荡24小时；

[0118]

③ Repeat steps ① and ② above three times.

---

③上述①、②两个步骤重复三次。

[0119]

Ultimately, a periosteum-bone complex with no cytotoxicity, low immunogenicity, good tissue activity, and a spatial composite structure was obtained.

最终获得无细胞毒性、低免疫原性、良好组织活性成分和空间复合结构的骨膜-骨复合体。

[0120]

Example 4: Periosteum-bone complex designed to reconstruct the immune repair environment of soft tissue and bone and its preparation method

---

实施例4：旨在重建软组织-骨免疫修复环境的骨膜-骨复合体及其制备方法

[0121]

(1)Acquisition of materials

---

(1)取材

[0122]

Take the femur from a healthy adult Large White pig slaughtered within 12 hours, separate the metaphysis, collect the femoral shaft, remove the internal medullary cavity, and cut the femoral shaft into 10mm*8mm pieces.

取宰杀12h以内健康成年大白猪的股骨，分离干骺端，收集股骨干部分，去除内部骨髓腔，将股骨干分割成10mm*8mm片状；

[0123]

(2) Pretreatment

---

(2)预处理

[0124]

Take a certain amount of bone slices, place them on a low-temperature (4°C) shaker at 100 rpm, and rinse them three times with PBS buffer containing protease inhibitors for 10 min each time to remove blood, attached fat fragments and other impurities.

取一定量的片状骨，置于低温(4°C)摇床100rpm震荡，用含蛋白酶抑制剂的PBS缓冲液漂洗3次，每次10min，去除血液、贴附的脂肪碎片和其他杂质；

[0125]

(3) Decalcification

---

(3)脱钙

[0126]

The pretreated bone sheets were placed in a 20% (w/v) EDTA-2Na solution and ultrasonically decalcified for 12 days. The ultrasonic parameters were 40 kHz and the temperature was 22 ° C.

将上述预处理的片状骨置于20%(w/v)EDTA-2Na溶液中超声脱钙12天，超声参数为40kHZ，温度22℃；

[0127]

(4) Obtaining the periosteal-bone complex

---

(4)骨膜-骨复合体获取

[0128]

① Three cycles of liquid nitrogen freeze-thaw (-80°C/37°C);

---

①液氮冻融3个循环(-80°C/37°C)；

[0129]

② Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 2% Triton X-100 PBS buffer, and shake at 100 rpm for 12 hours at low temperature (4°C);

---

②在浓度为2%的Triton X-100的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡12小时；

[0130]

③ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 0.5% SLES-containing deionized water, and shake at 100rpm for 4 hours at low temperature (4°C);

---

③在浓度为0.5%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡4小时；

[0131]

④ Wrap the periosteum portion of the periosteum-bone complex with sealing film to minimize its external exposure;

---

④用封口膜具包裹骨膜-骨复合体的骨膜部分，使其尽可能不对外暴露；

[0132]

⑤ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 0.5% SLES-containing deionized water and shake at 100 rpm for 24 hours at low temperature (4°C).

---

⑤在浓度为0.5%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡24小时；

[0133]

⑥ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1mg/ml PBS buffer containing DNase I, and shake at 100rpm for 12 hours at 37°C to obtain the periosteal bone scaffold.

---

⑥在浓度为1mg/ml的含DNase I的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，37°C摇床100rpm震荡12小时，即可得到骨膜骨支架。

[0134]

(5) Detoxification of the periosteal-bone complex

---

(5)骨膜-骨复合体脱毒

[0135]

① In Tris-HCl buffer, shake at 100 rpm on a low temperature (4°C) for 24 hours;

---

①在三羟甲基氨基甲烷盐酸盐(Tris-HCl)缓冲液中，低温(4°C)摇床100rpm震荡24小时；

[0136]

② Add the mixed antibacterial solution to sterile physiological saline and shake at 100 rpm for 12 hours at low temperature (4°C);

---

②在无菌生理盐水中，加入混合抗菌溶液，低温(4°C)摇床100rpm震荡12小时；

[0137]

③ Repeat steps ① and ② above three times.

---

③上述①、②两个步骤重复三次。

[0138]

Ultimately, a periosteum-bone complex with no cytotoxicity, low immunogenicity, good tissue activity, and a spatial composite structure was obtained.

最终获得无细胞毒性、低免疫原性、良好组织活性成分和空间复合结构的骨膜-骨复合体。

[0139]

Example 5: Periosteum-bone complex and its preparation method for reconstructing the soft tissue-bone immune repair environment.

实施例5：旨在重建软组织-骨免疫修复环境的骨膜-骨复合体及其制备方法

[0140]

(1)Acquisition of materials

---

(1)取材

[0141]

Take the femur from a healthy adult Large White pig slaughtered within 12 hours, separate the metaphysis, collect the femoral shaft, remove the internal medullary cavity, and cut the femoral shaft into 10mm*8mm pieces.

取宰杀12h以内健康成年大白猪的股骨，分离干骺端，收集股骨干部分，去除内部骨髓腔，将股骨干分割成10mm*8mm片状；

[0142]

(2) Pretreatment

---

(2)预处理

[0143]

Take a certain amount of bone slices, place them on a low-temperature (4°C) shaker at 100 rpm, and rinse them three times with PBS buffer containing protease inhibitors for 10 min each time to remove blood, attached fat fragments and other impurities.

取一定量的片状骨，置于低温(4°C)摇床100rpm震荡，用含蛋白酶抑制剂的PBS缓冲液漂洗3次，每次10min，去除血液、贴附的脂肪碎片和其他杂质；

[0144]

(3) Decalcification

---

(3)脱钙

[0145]

The pretreated bone sheets were placed in a 20% (w/v) EDTA-2Na solution and ultrasonically decalcified for 12 days. The ultrasonic parameters were 40 kHz and the temperature was 22 ° C.

将上述预处理的片状骨置于20%(w/v)EDTA-2Na溶液中超声脱钙12天，超声参数为40kHZ，温度22°C；

[0146]

(4) Obtaining the periosteal-bone complex

---

(4)骨膜-骨复合体获取

[0147]

① Three cycles of liquid nitrogen freeze-thaw (-80°C/37°C);

---

①液氮冻融3个循环(-80°C/37°C)；

[0148]

② Add penicillin and streptomycin (20 U/ml, 20 μg/ml) mixed in a 1:1 ratio to 0.5% Triton X-100 PBS buffer and shake at 100 rpm for 24 hours at low temperature (4°C).

---

②在浓度为0.5%的Triton X-100的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡24小时；

[0149]

③ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 0.5% SLES-containing deionized water, and shake at 100rpm for 4 hours at low temperature (4°C);

---

③在浓度为0.5%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡4小时；

[0150]

④ Wrap the periosteum portion of the periosteum-bone complex with sealing film to minimize its external exposure;

---

④用封口膜具包裹骨膜-骨复合体的骨膜部分，使其尽可能不对外暴露；

[0151]

⑤ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 0.5% SLES-containing deionized water and shake at 100 rpm for 24 hours at low temperature (4°C).

---

⑤在浓度为0.5%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡24小时；

[0152]

⑥ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1mg/ml PBS buffer containing DNase I, and shake at 100rpm for 12 hours at 37°C to obtain the periosteal bone scaffold.

---

⑥在浓度为1mg/ml的含DNase I的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，37°C摇床100rpm震荡12小时，即可得到骨膜骨支架。

[0153]

(5) Detoxification of the periosteal-bone complex

---

(5)骨膜-骨复合体脱毒

[0154]

① In Tris-HCl buffer, shake at 100 rpm on a low temperature (4°C) for 24 hours;

---

①在三羟甲基氨基甲烷盐酸盐(Tris-HCl)缓冲液中，低温(4°C)摇床100rpm震荡24小时；

[0155]

② Add the mixed antibacterial solution to sterile physiological saline and shake at 100 rpm for 24 hours at low temperature (4°C);

---

②在无菌生理盐水中，加入混合抗菌溶液，低温(4°C)摇床100rpm震荡24小时；

[0156]

③ Repeat steps ① and ② above three times.

---

③上述①、②两个步骤重复三次。

[0157]

Ultimately, a periosteum-bone complex with no cytotoxicity, low immunogenicity, good tissue activity, and a spatial composite structure was obtained.

最终获得无细胞毒性、低免疫原性、良好组织活性成分和空间复合结构的骨膜-骨复合体。

[0158]

Example 6: Periosteum-bone complex designed to reconstruct the immune repair environment of soft tissue and bone and its preparation method

---

实施例6：旨在重建软组织-骨免疫修复环境的骨膜-骨复合体及其制备方法

[0159]

(1)Acquisition of materials

---

(1)取材

[0160]

Take the femur from a healthy adult Large White pig slaughtered within 12 hours, separate the metaphysis, collect the femoral shaft, remove the internal medullary cavity, and cut the femoral shaft into 10mm*8mm pieces.

取宰杀12h以内健康成年大白猪的股骨，分离干骺端，收集股骨干部分，去除内部骨髓腔，将股骨干分割成10mm*8mm片状；

[0161]

(2) Pretreatment

---

(2)预处理

[0162]

Take a certain amount of bone slices, place them on a low-temperature (4°C) shaker at 100 rpm, and rinse them three times with PBS buffer containing protease inhibitors for 10 min each time to remove blood, attached fat fragments and other impurities.

取一定量的片状骨，置于低温(4°C)摇床100rpm震荡，用含蛋白酶抑制剂的PBS缓冲液漂洗3次，每次10min，去除血液、贴附的脂肪碎片和其他杂质；

[0163]

(3) Decalcification

---

(3)脱钙

[0164]

The pretreated bone sheets were placed in a 20% (w/v) EDTA-2Na solution and ultrasonically decalcified for 12 days. The ultrasonic parameters were 40 kHz and the temperature was 22 °C.

将上述预处理的片状骨置于20%(w/v)EDTA-2Na溶液中超声脱钙12天，超声参数为40kHZ，温度22°C；

[0165]

(4) Obtaining the periosteal-bone complex

---

(4)骨膜-骨复合体获取

[0166]

① Three cycles of liquid nitrogen freeze-thaw (-80°C/37°C);

---

①液氮冻融3个循环(-80°C/37°C)；

[0167]

② Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to PBS buffer containing 1% Triton X-100, and shake at 100 rpm for 12 hours at low temperature (4°C);

---

②在浓度为1%的Triton X-100的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡12小时；

[0168]

③ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1% deionized water containing SLES, and shake at 100rpm for 4 hours at low temperature (4°C);

---

③在浓度为1%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡4小时；

[0169]

④ Wrap the periosteum portion of the periosteum-bone complex with sealing film to minimize its external exposure;

---

④用封口膜具包裹骨膜-骨复合体的骨膜部分，使其尽可能不对外暴露；

[0170]

⑤ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1% deionized water containing SLES, and shake at 100rpm for 24 hours at low temperature (4°C).

---

⑤在浓度为1%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡24小时；

[0171]

⑥ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution at a ratio of 1:1 to 2mg/ml PBS buffer containing DNase I, and shake at 100rpm for 12 hours at 37°C to obtain the periosteal bone scaffold.

---

⑥在浓度为2mg/ml的含DNase I的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，37°C摇床100rpm震荡12小时，即可得到骨膜骨支架。

[0172]

(5) Detoxification of the periosteal-bone complex

---

(5)骨膜-骨复合体脱毒

[0173]

① In Tris-HCl buffer, shake at 100 rpm on a low temperature (4°C) for 24 hours;

---

①在三羟甲基氨基甲烷盐酸盐(Tris-HCl)缓冲液中，低温(4°C)摇床100rpm震荡24小时；

[0174]

② Add the mixed antibacterial solution to sterile physiological saline and shake at 100 rpm for 12 hours at low temperature (4°C);

---

②在无菌生理盐水中，加入混合抗菌溶液，低温(4°C)摇床100rpm震荡12小时；

[0175]

③ Repeat steps ① and ② above three times.

---

③上述①、②两个步骤重复三次。

[0176]

Ultimately, a periosteum-bone complex with no cytotoxicity, low immunogenicity, good tissue activity, and a spatial composite structure was obtained.

最终获得无细胞毒性、低免疫原性、良好组织活性成分和空间复合结构的骨膜-骨复合体。

[0177]

Example 7: Periosteum-bone complex designed to reconstruct the immune repair environment of soft tissue and bone and its preparation method

---

实施例7：旨在重建软组织-骨免疫修复环境的骨膜-骨复合体及其制备方法

[0178]

(1)Acquisition of materials

---

(1)取材

[0179]

Take the femur from a healthy adult Large White pig slaughtered within 12 hours, separate the metaphysis, collect the femoral shaft, remove the internal medullary cavity, and cut the femoral shaft into 10mm*8mm pieces.

取宰杀12h以内健康成年大白猪的股骨，分离干骺端，收集股骨干部分，去除内部骨髓腔，将股骨干分割成10mm*8mm片状；

[0180]

(2) Pretreatment

---

(2)预处理

[0181]

Take a certain amount of bone slices, place them on a low-temperature (4°C) shaker at 100 rpm, and rinse them three times with PBS buffer containing protease inhibitors for 10 min each time to remove blood, attached fat fragments and other impurities.

取一定量的片状骨，置于低温(4°C)摇床100rpm震荡，用含蛋白酶抑制剂的PBS缓冲液漂洗3次，每次10min，去除血液、贴附的脂肪碎片和其他杂质；

[0182]

(3) Decalcification

---

(3)脱钙

[0183]

The pretreated bone sheets were placed in a 20% (w/v) EDTA-2Na solution and ultrasonically decalcified for 12 days. The ultrasonic parameters were 40 kHz and the temperature was 22 ° C.

将上述预处理的片状骨置于20%(w/v)EDTA-2Na溶液中超声脱钙12天，超声参数为40kHZ，温度22°C；

[0184]

(4) Obtaining the periosteal-bone complex

---

(4)骨膜-骨复合体获取

[0185]

① Three cycles of liquid nitrogen freeze-thaw (-80°C/37°C);

---

①液氮冻融3个循环(-80°C/37°C)；

[0186]

② Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to PBS buffer containing 1% Triton X-100, and shake at 100 rpm for 12 hours at low temperature (4°C);

---

②在浓度为1%的Triton X-100的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡12小时；

[0187]

③ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1% deionized water containing SLES, and shake at 100rpm for 4 hours at low temperature (4°C);

---

③在浓度为1%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡4小时；

[0188]

④ Wrap the periosteum portion of the periosteum-bone complex with sealing film to minimize its external exposure;

---

④用封口膜具包裹骨膜-骨复合体的骨膜部分，使其尽可能不对外暴露；

[0189]

⑤ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution in a 1:1 ratio to 1% deionized water containing SLES, and shake at 100rpm for 24 hours at low temperature (4°C).

---

⑤在浓度为1%的含SLES的去离子水中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，低温(4°C)摇床100rpm震荡24小时；

[0190]

⑥ Add penicillin and streptomycin (20U/ml, 20μg/ml) mixed antibacterial solution at a ratio of 1:1 to 2mg/ml PBS buffer containing DNase I, and shake at 100rpm for 12 hours at 37°C to obtain the periosteal bone scaffold.

---

⑥在浓度为2mg/ml的含DNase I的PBS缓冲液中，1：1加入青霉素和链霉素(20U/ml，20μg/ml)混合抗菌液，37°C摇床100rpm震荡12小时，即可得到骨膜骨支架。

[0191]

(5) Detoxification of the periosteal-bone complex

---

(5)骨膜-骨复合体脱毒

[0192]

① In Tris-HCl buffer, shake at 100 rpm on a low temperature (4°C) for 24 hours;

---

①在三羟甲基氨基甲烷盐酸盐(Tris-HCl)缓冲液中，低温(4°C)摇床100rpm震荡24小时；

[0193]

② Add the mixed antibacterial solution to sterile physiological saline and shake at 100 rpm for 24 hours at low temperature (4°C);

---

②在无菌生理盐水中，加入混合抗菌溶液，低温(4°C)摇床100rpm震荡24小时；

[0194]

③ Repeat steps ① and ② above three times.

---

③上述①、②两个步骤重复三次。

[0195]

Ultimately, a periosteum-bone complex with no cytotoxicity, low immunogenicity, good tissue activity, and a spatial composite structure was obtained.

最终获得无细胞毒性、低免疫原性、良好组织活性成分和空间复合结构的骨膜-骨复合体。

[0196]

Examples 2-7 all successfully prepared decellularized periosteal-bone complex materials with complete decellularization, intact active ingredients, and complete three-dimensional structure, with results that were basically consistent with those of Example 1.

实施例2-7均能制备出脱细胞完全、活性成分保留完好和空间三维结构完整的骨膜-骨复合体脱细胞材料，相应结果与实施例1基本一致。