Notice

DESCRIPTION CN104511052A

A method for culturing a composite of periosteal bioscaffold and allogeneic seed cells.

一种骨膜生物支架与异体种子细胞复合的培养方法

[0001]

Technical Field

技术领域

[0002]

This invention belongs to the field of bioengineering technology, specifically relating to a method for culturing a combination of periosteal biological scaffold and allogeneic seed cells.

本发明属于生物工程技术领域，具体涉及一种骨膜生物支架与异 体种子细胞复合的培养方法。

[0003]

Background Technology

背景技术

[0004]

The periosteum has a strong osteogenic capacity and plays an important role in fracture healing and repair. The periosteum is also considered to be the initiating factor that promotes the repair function of bone graft materials in the transplantation area.

骨膜具有较强的成骨能力,在骨折愈合修复方面扮演着重要角 色，骨膜也被认为是促进骨移植材料在移植区域发挥修复作用的始动 因素。

However, autologous periosteum transplantation has problems such as insufficient donors and damage to the structure and function of the donor site, while allogeneic periosteum transplantation has problems such as severe immune rejection.

然而自体骨膜移植存在供体不足、供区结构功能损害等问题， 而异体骨膜移植则存在严重的免疫排斥反应等问题。

Therefore, addressing the issue of immune rejection in allogeneic periosteum will be a crucial step in achieving periosteum transplantation.

因此解决异体骨 膜中的免疫排斥问题将是实现骨膜移植的重要环节。

Cells are the main immunogenic component of tissues, and removing cells also means removing the immunogenic component. Currently, the main methods for removing cells include physical methods, chemical methods, and enzymatic methods.

组织中具有主要 免疫源性的成份为细胞，在去除细胞的同时也意味着免疫源性的去 除。 目前去除细胞的方法主要包括物理方法、化学方法和酶法。

[0005]

While the periosteum can effectively remove immunogenic cellular components through physical, chemical, and related enzymatic processes, the treatment inevitably damages the composition and structure of the extracellular matrix, thereby affecting the biocompatibility of the scaffold and making it difficult for cells to integrate with it.

骨膜组织通过物理、化学及相关酶的作用后虽然可以有效去除具 有免疫源性的细胞成份，然而处理过程中势必会对细胞外基质的成份 和结构造成不可避免的破坏，进而影响该支架的生物相容性，令细胞 难以与其复合。

Therefore, the ideal immunogenic-free periosteal bioscaffold obtained through optimized deimmunogenic treatment should remove cellular components while preserving as completely as possible the main bioactive components and structures of the scaffold's extracellular matrix, providing a suitable and natural growth space for the ingrowth of allogeneic seed cells. On the other hand, scaffold materials and seed cells are two essential core components in bone tissue engineering. The combination of scaffold materials with seed cells in vitro can ensure that they have a stronger repair and reconstruction capacity after being implanted at the target site.

因此，通过优化脱免疫源性的处理所获取的理想的无 免疫源性骨膜生物支架应是在去除细胞成份的同时又尽可能完整地 保留支架细胞外基质的主要生物活性成份及结构，为异体种子细胞地 长入提供

一个合适的、天然的生长空间。 另一方面，支架材料和种子 细胞是骨组织工程中必不可少的两个核心环节，支架材料在体外复合 种子细胞可以保证其植入靶位置后具有更强的修复重建能力。

[0006]

Summary of the Invention

发明内容

[0007]

This invention provides a method for culturing a composite periosteal biological scaffold with allogeneic seed cells, with the aim of preparing a tissue engineering scaffold composed of composite periosteal cells, providing a biocomposite scaffold material for tissue engineering research on bone defects and non-union.

本发明提供了一种骨膜生物支架与异体种子细胞复合的培养方 法，目的在于制备复合骨膜细胞的组织工程支架，为骨缺损、骨不愈 的组织工程研究提供生物复合支架材料。

[0008]

This invention is specifically achieved through the following technical solutions:

本发明具体通过以下技术方案实现：

[0009]

A method for culturing a composite of periosteal bioscaffold and allogeneic seed cells, comprising the following steps:

一种骨膜生物支架与异体种子细胞复合的培养方法，该方法包括 以下步骤：

[0010]

1) Preparation and identification of non-immunogenic periosteal bioscaffolds;

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1)无免疫源性骨膜生物支架的制备和鉴定；

[0011]

2) After the preserved non-immunogenic periosteal biological scaffold is dried at low temperature, it is sealed and sterilized with gamma rays;

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2)将保存的无免疫源性骨膜生物支架低温干燥后密封，使用γ 射线消毒；

[0012]

3) Take allogeneic periosteum tissue, cut it into small pieces, digest it with collagenase for 4 hours, centrifuge the supernatant, collect the cells, and culture them in a culture flask to obtain seed periosteum cells;

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3)取异体骨膜组织剪成碎块，用胶原酶消化4h，取上清液离心 后收集细胞，放于培养瓶中培养，获得种子骨膜细胞；

[0013]

4) Drop seed periosteal cells at a concentration of 1×10^{1} NER^{1}/ml onto the surface of a sterilized scaffold. After the periosteal cells gradually infiltrate into the non-immunogenic periosteal biological scaffold with the suspension, add culture medium and change the medium every 2-3 days for 1 week.

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4)将种子骨膜细胞以浓度为1×10 ⁶ /ml，滴于消毒的支架表面， 待骨膜细胞逐渐随着悬液渗入无免疫源性骨膜生物支架内部后添加 培养基，每2～3天换液，培养1周。

[0014]

further:

进一步：

[0015]

Step (1) specifically includes the following steps:

步骤(1)具体为包括以下步骤：

[0016]

a.

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a.

The periosteum was rinsed, frozen at -80°C for 24–48 hours, and then thawed at 37°C. This process was repeated 3–5 times.

将骨膜组织冲洗，在-80°C条件下冷冻24～48h，取出后37°C条 件下融化，重复3～5次；

[0017]

b.

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b.

The cell-decellularizing solution was shaken for 12 h in 3% Triton-X100 and 3.5 × 10^2 mol/L benzyl xanthyl fluoride (PMSF), repeatedly washed with PBS, and then placed in 1% sodium dodecyl sulfate (SDS) and shaken for another 6 h.

在3%曲拉通-X100(Triton-X100)和3.5×10 ⁻⁵ mol/L苯甲基黄 酰氟(PMSF)的脱细胞液震荡12h，PBS反复冲洗并放入1%十二烷 基硫酸钠(SDS)中继续震荡6h；

[0018]

c.

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c.

After rinsing, place the periosteum tissue in a mixed digestive enzyme solution containing 0.001 U/L RNase and 0.1 U/L DNase for 30–40 minutes;

冲洗后将骨膜组织放于混有0.001U/L RNA酶和0.1U/L DNA酶 的混合消化酶液中30～40min；

[0019]

d.

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d.

The periosteal bioscaffold was sterilized by immersion in 1% peracetic acid solution for 4 hours to obtain an immunogenic bioscaffold. It was then stored in PBS containing 0.1 U/L penicillin and streptomycin at 4°C for later use.

在体积分数1%的过氧乙酸溶液浸泡消毒4h，既得无免疫源性 骨膜生物支架，置于含0.1U/L的青链霉素的PBS液中4°C环境下保 存备用。

[0020]

In step (2), gamma rays are used for disinfection, with a total irradiation dose of 15 KGray.

步骤(2)中使用γ射线消毒，照射总剂量为15KGray。

[0021]

The collagenase mentioned in step (3) is type I collagenase at 2 g/L, and the culture flask contains DMEM/F12 medium containing 10% fetal bovine serum and 1% penicillin and streptomycin.

步骤(3)所述的胶原酶为2g/L的I型胶原酶，所述的培养瓶内 含10%胎牛血清和1%青链霉素的DMEM/F12培养基。

[0022]

The culture medium described in step (4) is DMEM/F12 medium containing 10% fetal bovine serum and 1% penicillin and streptomycin.

步骤(4)所述的培养基为含10%胎牛血清和1%青链霉素的 DMEM/F12培养基。

[0023]

The beneficial effects of this invention are that the immunogenic cell structures in the periosteum bioscaffold obtained by methods such as physical freeze-thaw, detergent elution and enzyme digestion are thoroughly removed, while the main structure and components of the extracellular matrix are well preserved, and the allogeneic periosteum cells used can be effectively combined with the periosteum bioscaffold.

本发明的有益效果为运用物理冻融、去污剂洗脱和酶消化等方法 所获取的骨膜生物支架中含免疫源性的细胞结构去除彻底，细胞外基 质的主要结构及成分保留完好，所用的异体骨膜细胞可以与该骨膜生 物支架有效复合。

[0024]

Attached Figure Description

附图说明

[0025]

Figure 1 shows the histological staining of normal periosteum; a) DAPI staining; b) HE staining; c) Masson staining.

图1是正常骨膜组织学染色观察图；a为DAPI染色；b为HE 染色；c为Masson染色；

[0026]

Figure 2 shows histological staining of non-immunogenic periosteum; a) DAPI staining; b) HE staining; c) Masson staining.

图2是无免疫源性骨膜组织学染色观察图；a为DAPI染色；b 为HE染色；c为Masson染色；

[0027]

Figure 3 shows the scanning electron microscope (SEM) images of the periosteum; a is the SEM image of normal periosteum; b is the SEM image of periosteum without immunogenicity.

图3是骨膜扫描电镜观察图；a为正常骨膜扫描电镜观察图；b 为无免疫源性骨膜扫描电镜观察图；

[0028]

Figure 4 shows the histological examination of the composite scaffold; a) HE staining to observe the co-culture of cells and scaffold; b) fluorescence observation of cell co-culture on the scaffold.

图4是复合支架组织学检查图；a为HE染色观察细胞与支架复 合培养情况；b为荧光下观察细胞在支架上的复合情况。

[0029]

Detailed Implementation

具体实施方式

[0030]

The present invention will be further described below with reference to the embodiments. The following description is only a preferred embodiment of the present invention and is not intended to limit the present invention in other ways. Any person skilled in the art may make changes to the above-disclosed technical content to create equivalent embodiments with the same changes.

下面结合实施例对本发明做进一步的说明，以下所述，仅是对本 发明的较佳实施例而已，并非对本发明做其他形式的限制，任何熟悉 本专业的技术人员可能利用上述揭示的技术内容加以变更为同等变 化的等效实施例。

Any simple modifications or equivalent changes made to the above embodiments without departing from the scope of the present invention and based on the technical essence of the present invention shall fall within the protection scope of the present invention.

凡是未脱离本发明方案内容，依据本发明的技术实 质对以上实施例所做的任何简单修改或等同变化，均落在本发明的保 护范围内。

[0031]

Example 1: A method for culturing rabbit tibia non-immunogenic periosteal bioscaffold combined with seed cells.

实施例1兔胫骨无免疫源性骨膜生物支架与种子细胞复合的培 养方法

[0032]

1. Materials

1、材料

[0033]

Laboratory animals: Healthy New Zealand White rabbits, provided by the Animal Experiment Center of Wenzhou Medical University, 3-4 months old, weighing approximately 2.0-2.5 kg, male or female, fed with regular diet and water, and housed in separate cages.

实验动物：为健康新西兰大白兔，由温州医科大学动物实验中心 提供，3～4月龄，体质量约为2.0～2.5kg，雌雄不限，常规饮食饮水， 分笼饲养。

[0034]

Main reagents and instruments: Triton X100 (Sigma-Aldrich), sodium dodecyl sulfate (SDS, Sigma-Aldrich), PMSF (Sigma-Aldrich), RNase, DNase (Sigma-Aldrich), type I collagenase (GIBCO), DNA extraction kit (TAKARA, Dalian), hydroxyproline (Hyp) test kit (Nanjing Kaiji

Biotechnology), DAPI staining solution (Sigma-Aldrich), CCK-8 (Dōjin, Japan), fetal bovine serum (GIBCO, USA), penicillin, streptomycin (Shanghai Boyun Biotechnology Co., Ltd.), DMEM /F-12 culture medium (GIBCO, USA), constant temperature shaker (Heidolph, Germany), micro-volume UV spectrophotometer (NanoDrop, USA), CX31-32RFL fluorescence microscope (OLYMPUS, Japan). The equipment included: an S-3000N scanning electron microscope (Hitachi, Japan), an MRX-HD fully automated microplate reader (Biotek, USA), and a CO2 incubator (Hera-cell, Germany).

主要试剂及仪器设备：曲拉通-X100(Triton-X100，Sigma公 司)，十二烷基硫酸钠(SDS，美国Sigma公司)，苯甲基黄酰氟(PMSF， 美国Sigma公司)，RNA酶、DNA酶(美国Sigma公司)， Ⅰ型胶原 酶(美国GIBCO公司)，DNA提取试剂盒(大连TAKARA公司)， 羟脯氨酸(Hyp)测试盒(南京凯基生物)，DAPI染液(美国Sigma 公司)，CCK-8(日本同仁)，胎牛血清(美国GIBCO公司)，青霉素、 链霉素(上海博蕴生物有限公司)，DMEM/F-12培养基(美国GIBCO 公司)，恒温震荡机(德国Heidolph公司)、微量紫外分光光度仪(美 国NanoDrop公司)、CX31-32RFL荧光显微镜(日本OLYMPUS公 司)、S-3000N型扫描电子显微镜(日本Hitachi公司)、MRX-HD全 自动酶标分析仪(美国Biotek公司)、CO2培养箱(德国Hera-cell 公司)。

[0035]

2. Preparation of non-immunogenic periosteal bioscaffold materials

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2、无免疫源性骨膜生物支架材料的制备

[0036]

2.1 Separation and sampling of rabbit tibial periosteum

2.1兔胫骨骨膜的分离取材

[0037]

After anesthesia with 1 ml/kg sodium pentobarbital (3% by volume) via the marginal ear vein, the periosteum was dissected on the medial side of the proximal tibia on both sides.

予体积分数为3%的戊巴比妥钠1ml/kg耳缘静脉麻醉后，于双侧 胫骨近端内侧分离骨膜。

During the procedure, the soft tissues such as muscles on the surface of the periosteum are carefully removed. A 1.5cm × 1.5cm section of the full-thickness periosteum is then cut off with a sterile scalpel and a periosteal elevator is used to slowly peel off the periosteum close to the surface of the bone cortex.

术中仔细剔除骨膜表面肌肉等软组织，用无 菌手术刀片切取1.5cm×1.5cm范围的骨膜全层后使用骨膜剥离器紧 贴骨皮质表面缓慢剥下骨膜。

Throughout the entire separation and sampling process, the surgical field should be kept as clean as possible, and the periosteum should be kept intact.

整个分离取材过程尽量保持术野清洁， 骨膜组织保持形态完整。

[0038]

2.2 Removal process of periosteal immunogenic components

2.2骨膜免疫源性成份去除过程

[0039]

The extracted free periosteum was rinsed repeatedly with PBS 3-4 times to remove residual blood and other impurities. After freezing at -80°C for 24-48 hours, it was thawed at 37°C (repeated 5 times). Then, it was placed in a decellularization solution containing 3% Triton-X100 and 3.5×10^{3} mol/L PMSF and shaken at 37°C for 12 hours. After that, it was rinsed repeatedly with PBS and then placed in 1% SDS and shaken for another 6 hours.

将取出的游离骨膜用PBS反复冲洗3-4次，去除组织上残留的血 液及其他杂质，放入-80°C冰箱内冷冻24-48小时后取出后37°C条件 下融化(反复5个循环)后，放入含3%Triton-X100和3.5×10 <sup>

-5 </sup> mol/L PMSF的脱细胞液中置于37°C恒温震荡机上震荡12小时，后予PBS 反复冲洗并放入1%SDS中继续震荡6小时。

After rinsing, the periosteum is placed in a mixed digestive enzyme solution containing 0.001 U/L RNase and 0.1 U/L DNase for 30–40 minutes, then soaked in 1% peracetic acid solution for 4 hours for disinfection. After rinsing thoroughly with PBS 3 times, it is stored in PBS solution containing 0.1 U/L penicillin and streptomycin at 4°C for later use.

冲洗后将骨膜放于混有 0.001U/L RNA酶和0.1U/L DNA酶的混合消化酶液中30～40分钟后, 予体积分数1%过氧乙酸溶液浸泡消毒4小时，PBS充分冲洗3遍后 置于含0.1U/L的青链霉素的PBS液中4°C环境下保存备用。

[0040]

3. Identification of non-immunogenic periosteal bioscaffolds

3、无免疫源性骨膜生物支架的鉴定

[0041]

3.1 General observation

3.1大体观察

[0042]

Fresh periosteal tissue and tissue treated with physical freeze-thaw cycles, Triton-X100, SDS shaking, and mixed digestive enzyme solutions including RNase and DNase showed that the periosteal tissue exhibited a white, loose, spongy structure.

观察新鲜骨膜组织及经物理冻融，Triton-X100、SDS震荡作用及 RNA酶、DNA酶等混合消化酶液处理后，骨膜组织呈现白色疏松海 绵状结构。

[0043]

3.2 Histological examination

3.2组织学检查

[0044]

The periosteum of the normal group and after deimmunogenic treatment was fixed with 4% paraformaldehyde, dehydrated with alcohol gradient, cleared with xylene, embedded in paraffin, sectioned, and routinely stained with HE, DAPI and Masson stains for observation. As shown in Figures 1 and 2, the immunogenic cells in the periosteum tissue were completely

removed after treatment, while the main components of the extracellular matrix (collagen) were effectively preserved.

正常组及脱免疫源性处理后的骨膜，经4%多聚甲醛固定，酒精 梯度脱水，二甲苯透明，浸蜡、包埋、切片，常规行组织学HE染色、 DAPI染色和Masson染色后观察，如图1和图2所示，经处理后骨膜组织中具有免疫源性的细胞已完全去除，而细胞外基质中的主要成 份(胶原)则有效保留。

[0045]

3.3 Quantitative detection of collagen

3.3胶原定量检测

[0046]

The hydroxyproline measurement method was used to estimate the change in collagen content before and after deimmunogenic treatment, based on the fact that hydroxyproline accounts for 13.4% of collagen.

采用羟脯氨酸测量法，根据羟脯氨酸在胶原蛋白中含量占13.4% 以推算脱免疫源性处理前后胶原含量的变化。

The specific procedure for the hydroxyproline test kit (Kaiji Biotechnology) is as follows: Accurately weigh 30-100 mg of normal periosteal tissue (n=6) and processed periosteal tissue (n=6) into a test tube and add hydrolysis solution. After boiling in a water bath for 20 minutes, adjust the pH to approximately 6.0-6.8. After repeated centrifugation and purification, the sample and standard solution are tested for absorbance at 550 nm using an ELISA reader. The Hyp content per milligram of tissue is then calculated to further determine the collagen content of the periosteal tissue.

具体采用羟脯氨酸测试 盒(凯基生物)里说明书所提供的步骤操作：精确称取正常骨膜组织 (n＝6)和处理后骨膜组织(n＝6)湿重30～100mg放入试管中并加入 水解液，沸水浴20分钟后，调节PH值至6.0～6.8左右，经反复离心、 纯化后和标准液一同予550nm的酶标仪检测吸光度并计算每毫克组 织Hyp含量以进一步得出骨膜组织的胶原含量。

The results showed no statistically significant difference in collagen content between the non-immunogenic periosteal bioscaffold (34.72±1.29 μg/mg) and normal periosteal tissue (35.95±1.65 μg/mg) (P>0.05).

结果显示无免疫源 性骨膜生物支架(34.72±1.29μg/mg)与正常骨膜组织(35.95±1.65 μg/mg)胶原含量差别无统计学意义(P＞0.05)。

[0047]

3.4 Qualitative and quantitative analysis of DNA

3.4DNA定性定量分析

[0048]

The weight of the normal periosteum and the deimmunogenic periosteum was approximately 2–25 mg. After hydrolysis with proteinase K and RNase in a 56 °C water bath, genomic DNA was extracted (following the instructions of the Takara DNA kit). The concentration was determined by a micro-ultraviolet spectrophotometer, and the DNA content per milligram of tissue was calculated.

去正常骨膜及脱免疫源性处理后的骨膜质量约为2～25mg，在56 °C水浴中经蛋白酶K和RNA酶水解后，提取基因组DNA(按照Takara DNA试剂盒说明操作)，微量紫外分光光度仪测定其浓度,并计算每毫 克组织DNA含量。

The results showed that the DNA content of the non-immunogenic periosteal biological scaffold (32.4 ± 8.5 ng/mg) was significantly lower than that of normal periosteal tissue (1281.6 ± 631.6 ng/mg).

结果显示无免疫源性骨膜生物支架DNA含量(32.4 ±8.5ng/mg)较正常骨膜组织(1281.6±631.6ng/mg)明显减低。

[0049]

3.5 Scanning electron microscopy observation

3.5扫描电镜观察

[0050]

Normal group and periosteal tissue after immunogenic de-immunogenic treatment were fixed with 3% glutaraldehyde for 24 hours, washed with PBS, and dehydrated with a gradient of ethanol (volume fractions of 50%, 70%, 80%, 90%, 95%, and 100%) for more than 15 minutes each time. After vacuum drying and surface gold sputtering, the tissues were observed under a scanning electron microscope, as shown in Figure 3. The surface of the non-immunogenic periosteal bioscaffold showed a loose and porous structure, which is more conducive to cell aggregation.

正常组及脱免疫源性处理后的骨膜组织用3%戊二醛固定24小 时后，PBS冲洗，乙醇梯度(体积分数为50%、70%、80%、90%、 95%、100%)脱水，每个脱水15min以上，真空干燥，表面喷金处 理后在扫描电子显微镜下观察，如图3所示，见无免疫源性骨膜生物 支架表面呈疏松多孔结构，更有利于细胞复合。

[0051]

4. Isolation and culture of periosteal cells

4、骨膜细胞的分离和培养

[0052]

Under aseptic conditions, the free periosteum was removed, repeatedly rinsed with PBS, and then cut into approximately 1 mm pieces using ophthalmic scissors on a laminar flow hood. The pieces were digested with 2 g/L collagenase in a 37°C incubator containing 5% CO2 for 4 hours. The supernatant was collected, and the cells were collected by centrifugation at 1200 rpm for 5 minutes. The cells were then cultured in DMEM/F12 flasks containing 10% fetal bovine serum and 1% DMEM. When the cells reached the bottom of the flask, they were passaged, and the second-generation periosteum cells were collected for subsequent recombination.

无菌条件下取出游离骨膜，反复予PBS冲洗后在超净台上用眼 科剪将骨膜剪成lmm ³ 左右碎块，用2g/L的胶原酶放于37°C、含5 %CO2的培养箱中消化4h，取上清液，1200r/min离心5min离心收 集细胞，放于含10%胎牛血清和1%的DMEM/F12培养瓶中培养，当 细胞铺满瓶底时进行传代，收集第二代骨膜细胞进行后续复合。

[0053]

5. Periosteal cells and scaffold composite

5、骨膜细胞与支架复合

[0054]

Non-immunogenic periosteal biological scaffolds were dried at low temperature, sealed in a package, and sterilized using gamma rays (15KGray).

取无免疫源性骨膜生物支架低温干燥后密封包装,使用γ射线 (15KGray)消毒。

After sterilization, place the culture dish in a dry culture dish. Add the isolated second-generation periosteal cells at a concentration of 1×10 NER/ml to the surface of the scaffold. After the periosteal cells gradually infiltrate into the non-immunogenic periosteal biological scaffold with the suspension (3 hours), add culture medium. Change the medium every 2-3 days and culture for 1 week.

消毒后置于干燥培养皿中，将分离的第二代骨膜细 胞以浓度为1×10 ⁶ /ml，滴于支架表面，待骨膜细胞逐渐随着悬液渗 入无免疫源性骨膜生物支架内部(3小时)后添加培养基，每2-3天换液，培养1周。

[0055]

6. Histological examination of composite scaffolds

6、复合支架的组织学检查

[0056]

After 7 days of culture, the composite scaffold was washed with PBS and then Live-dead staining solution was added. The cell aggregation on the scaffold was observed under fluorescence. After fixation with 4% paraformaldehyde, dehydration with alcohol gradient, clearing with xylene, paraffin embedding, sectioning, and routine histological HE staining, the cell-scaffold aggregation culture was observed, as shown in Figure 4. The results showed that allogeneic periosteal cells could effectively aggregate and proliferate on the scaffold.

取培养7天后的复合支架予PBS清洗后添加Live-dead染液，荧 光下观察细胞在支架上的复合情况；经4%多聚甲醛固定，酒精梯度 脱水，二甲苯透明，浸蜡、包埋、切片，常规行组织学HE染色观察细胞与支架复合培养情况，如图4所示，结果均显示异体骨膜细胞可 在该支架上有效复合并增殖。