Complex of dog allogenic decalcified bone matrix and bone marrow stromal cell sheets***★

In vitro culture and observation

Tan Shuai, Jing Heng, Gao Zhen-hua, Li Ning-yi

Abstract

BACKGROUND: How to reconstruct tissue-engineered bone with structure similar to natural bone is a problem in the development of tissue engineering. Cell sheet engineering technology enables novel approaches to construction of tissue-engineered bone.

OBJECTIVE: To observe the biocompatibility of cell sheets to decalcified bone matrix (DBM) and their growth on DBM.

DESIGN, TIME AND SETTING: An *in vitro* observation was performed at the Central Laboratory, Affiliated Hospital, Qingdao University Medical College between June and September 2009.

MATERIALS: Dog bone marrow stromal cell sheets were prepared using temperature-responsive medium. Dog DBM was prepared by defatting, decalcification, and noncollagen protein removal procedures.

METHODS: DBM surface was covered by cell sheets prepared by temperature-responsive technology and cultured with DMEM containing 10% fetal bovine serum and osteoinductive agent.

MAIN OUTCOME MEASURES: Under scanning electron microscope, DBM structure, as well as the attachment and growth of cell sheets on DBM surface, was observed. Porosity and aperture size of DBM were calculated.

RESULTS: DBM exhibited a three-dimensional latticed structure, with a porosity of approximately 75%. The mean aperture size was (250.11±98.89) μm, exhibiting a normal distribution. Cell sheets well attached to and grew on DBM surface, and rapidly proliferated.

CONCLUSION: Cell sheets show good biocompatibility to DBM. DBM/cell sheets complex can be applied in tissue-engineered bones, which promotes the construction of tissue-engineered bone with structure similar to natural bone.

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INTRODUCTION

Research regarding tissue-engineered bone rapidly develops in recent years. How to construct tissue-engineered bone with structure similar to natural bone remains unclear, which has become a rapidly growing area of research. Cell sheet engineering technology enables novel approaches to tissue engineering and has attracted increasing attention. Strong evidence exists that cell sheet engineering technology has been used to successfully construct single cell sheets of cornea, periodontal ligament, skin, and urothelium and multiple cell sheets of myocardium, smooth muscle renal glomerulus, and hepatic lobule[1-4]. The present study used decalcified bone matrix (DBM) as scaffold to compound prepared cell sheets and observed the biocompatibility of cell sheets to DBM, as well as cell sheet growth on DBM.

MATERIALS AND METHODS

Design

An *in vitro* observation.

Time and setting

All experiments were performed at the Central Laboratory, Affiliated Hospital, Qingdao University Medical College between June and September 2009.

Materials

One 3-year-old healthy adult dog, weighing 25 kg, was provided by the Laboratory Animal Center, Affiliated Hospital, Qingdao University Medical College. The experimental protocol was in strict accordance with Animal Ethical Standards.

The primary materials and instruments are as follows:

Material/instrument	Source
Ultra low temperature freezer (MDF-382E)	SANYO, Japan
Lyophilizer (FD5508)	SIM, USA
Scanning electron microscope (JSM－840)	JEOL, Japan
CO_2 cell incubator(CH200)	Heto, Denmark
Anhydrous calcium chloride, anhydrous lithium chloride, acetone	Tianjin Chemical Regent Co., Ltd., China
Ethylenediamine tetraacetic acid (EDTA)	Sigma, USA

Methods

Culture of bone marrow stromal cells (BMSCs)

Dog BMSCs were isolated by density gradient centrifugation, proliferated, and induced to differentiate into osteoblasts[5-6].

Preparation of dog DBM

Following removal of soft tissue and periosteum, fresh hybrid dog femur was frozen at －80 ℃ for 6 months. A series of procedures including defatting, decalcification, noncollagen protein removal, and freeze-dried through the use of lyophilizer were performed to prepare allogenic DBM with a size of 2 cm × 2 cm × 5 mm[7].

Preparation of dog BMSC sheets

The induce-differentiated BMSCs were placed

on temperature-responsive medium (PIPAAm-TCPS) surface (Petri dish diameter 3.5 cm), and incubated with routine low-glucose DMEM medium at 37 ℃ in 5% CO_2 saturated humidity environment. When cells covered the whole bottle bottom and were confluent, they were incubated for 30 minutes at 20 ℃ in 5% CO_2 saturated humidity environment. Cell sheets, with a diameter of approximately 1.5–2.0 cm, were scraped through the use of cell scarper[8].

DBM structure observation and physical performance detection

DBM structure observation and aperture size determination: Dog DBM was made into 3 mm × 3 mm × 3 mm blocks for better observation of microstructure under scanning electron microscope. Five different regions were randomly selected for photographing. The aperture size of 100 random samples was measured using Image-Pro Plus 6.0 software, and the mean aperture size was calculated.

Porosity determination: DBM was thoroughly soaked in a volumetric cylinder, in which there was a certain volume of (V_1) of dehydrated alcohol. The total volume of DBM and dehydrated alcohol was V_2. After taking out the DBM, the volume of remaining dehydrated alcohol was V_3. Then, DBM porosity p =(V_1–V_3)/(V_2–V_3)×100%.

Construction of DBM/cell sheets complex

Six dog DBM blocks were prepared into the size satisfying for scanning electron microscope observation, and were sterilized with ethylene oxide for 48 hours for future use. DBM blocks were placed into a 6-well plate and soaked with DMEM for 48 hours. During this period, medium was replaced three times. The final pH value was 7.2–7.3. Another soaking procedure, in which DMEM containing 20% fetal bovine serum was utilized, was performed, followed by drying using sterile gauze. The scraped cell sheets were gently placed on DBM surface. DMEM containing 10% fetal bovine serum and osteoinductive agent was slowly dropped along the culture plate wall till the DBM was thoroughly soaked. This was followed by an incubation procedure at 37 ℃ in a 5% CO_2 saturated humidity environment.

Scanning electron microscope observation

At 3, 5, and 7 days after cell sheet inoculation, two complexes were taken out, rinsed three times with 0.01 mol/L PBS (pH 7.0), fixed with 2.5% glutaraldehyde for 12 hours, dehydrated in gradient acetone, treated with isoamyl acetate at room temperature for 2 hours, dried at critical point, gold-spread, and observed under scanning electron microscope. Finally, the attachment and growth of cell sheets on DBM were recorded.

Main outcome measures

DBM structure, porosity, and aperture size, and the attachment and growth of cell sheets on DBM surface.

Design, enforcement and evaluation

The fourth author designed this study. The first, second, and third authors collected data. The first author performed all experiments. The fourth author evaluated experimental data. Blind method evaluation was not employed.

RESULTS

DBM structure and physical performance

DBM exhibited a three-dimensional latticed structure (Figure 1), with a porosity of approximately 75%. The aperture size ranged between 89.4–454.2 μm, with a mean value of (250.11± 98.89) μm, exhibiting a normal distribution.

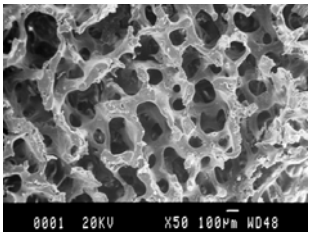

Figure 1 Scanning electron microscope image of decalcified bone matrix

Scanning electron microscope observation of DBM/cell sheets complex

Scanning electron microscope results showed that at 3 days after cell sheet inoculation, cell sheets attached to DBM and exhibited good cell activity (Figure 2). By day 5, cell sheets began to grow towards adjacent scaffold (Figure 3). By day 7, cell sheets grew rapidly and adjacent scaffold was a part covered by cell sheets (Figure 4).

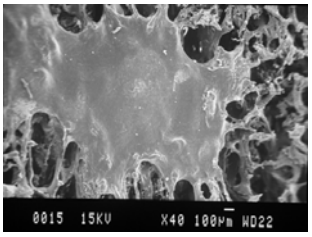

Figure 2 Scanning electron microscope image 3 days after inoculation of cell sheets

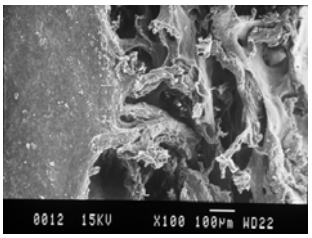

Figure 3 Scanning electron microscope image 5 days after inoculation of cell sheets

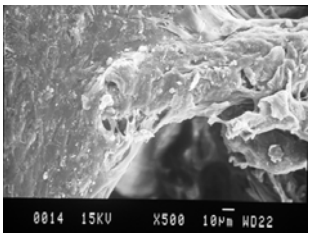

Figure 4 Scanning electron microscope image 7 days after inoculation of cell sheets

DISCUSSION

With the progress in research of tissue-engineered bone, how to construct large-scale tissue-engineered bone with structure similar to natural bone and stable blood supply has become a hot spot. The basic requirements are to construct clinically useful, large-scale, functional tissue-engineered bone with structure similar to natural bone.

Cell sheet engineering technology enables novel approaches to tissue-engineered bone with natural bone-like structure. This technology utilizes a theory, in which temperature-responsive medium is used, that temperature change makes poly(N-isopropylacrylamide) (PIPAAm) from hydrophobic into hydrophilic, thus cells cultured would separate from medium surface and form cell sheets and attach onto extracellular matrix[8-9]. Such cell sheets do not require proteolytic enzyme and cells would not be injured. The extracellular matrix at the bottom can directly form basal lamina to construct three-dimensional tissue structure. This cell inoculation method provides cells for bone formation and these cells exhibit the arrangement of osteoblasts during natural bone formation. Zhou *et al*[10] used this technology to successfully construct tissue-engineered bone with cortical bone- and spongy bone-like structure, which suggests the feasibility of this technology. Cell sheet engineering promotes the development of tissue-engineered bone, but there are some problems to be solved, for example, source of seed cells and control of cell sheet thickness[11].

Allogenic DBM has been shown to be a good biological scaffold material owing to its excellent biocompatibility and degradability, satisfactory mechanical strength, and easy sterilization[12]. Following defatting, decalcification, and noncollagen protein removal procedures, DBM exhibits low immunogenicity, reserves the growth factors and collagen for inducing bone formation. Its three-dimensional latticed structure provides good place for the attachment, proliferation, differentiation, osteogenesis, and vascularization of seed cells[13].

The present study constructed DBM/cell sheets complex. Results showed that cell sheets well attached to DBM, without obscission. During the process of construction, cell sheets were gently placed on DBM and 2.0–3.0 mL of culture medium was dropped. Thereafter, there was a 4.0–5.0 hours of culture procedure, which provides sufficient time for cell attachment. Finally, medium was slowly dropped along the culture plate wall, which contributes to good attachment. Scanning electron microscope results showed that at 3 days after inoculation, cell sheets well attached to DBM, showing satisfactory activity; at 5 days, cell sheets began to grow towards adjacent scaffolds; at 7 days, cell sheets rapidly grew towards adjacent scaffolds, and scaffold had be a part covered by cell sheets. These results demonstrate that DBM shows good biocompatibility to cell sheets and promotes cell proliferation and differentiation. DBM/cell sheet complex extremely promotes construction of tissue-engineered bone with structure similar to natural bone.

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犬同种异体脱钙骨基质复合细胞片层的体外培养与观察***

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摘要

背景：如何构建具有类似天然骨结构组织工程骨的问题是组织工程学发展的一个难题。细胞片层技术的出现，为其提供了新的途径。

目的：观察细胞片层与脱钙骨基质的生物相容性及其在脱钙骨基质上的生长情况。

设计、时间及地点：体外观察性实验，于 2009-06/2009-09 在青岛大学医学院附属医院中心实验室完成。

材料：利用温度感应培养基制备犬骨髓基质细胞片层；脱脂、脱钙、脱非胶原蛋白方法制备犬脱钙骨基质。

方法：将温度感应技术制备刮取的细胞片层铺盖于脱钙骨基质表面，用含体积分数为 10%胎牛血清及成骨诱导剂的 DMEM 培养液浸没培养。

主要观察指标：扫描电镜下观察脱钙骨基质的结构及细胞片层在脱钙骨基质上的附着、生长情况。计算脱钙骨基质孔隙率和孔径大小。

结果：扫描电镜下观察可见脱钙骨基质呈三维立体网孔结构。材料孔隙率约为 75%。平均孔隙直径为(250.11±98.89) μm，成正态性分布。扫描电镜下观察细胞片层在脱钙骨基质上的附着、生长状况良好，增殖迅速。

结论：细胞片层与脱钙骨基质有较好的生物相容性，脱钙骨基质/细胞片层复合体能够应用于组织工程骨，为构建类似骨结构组织工程骨起到促进作用。

关键词：脱钙骨基质；细胞片层；组织工程；犬；生物材料
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[http://www.crter.org　http://cn.zglckf.com]
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关于生物材料研究有价值的近期新闻回顾：本刊学术部

内容简介	网站点击更多
2009 年 6 月，由天津大学材料科学与工程学院蔡舒教授等人承担的天津市自然科学基金项目“新型仿生骨”生物材料技术完成实验室阶段研究，并将应用于医疗领域。由新型材料制作的“仿生骨”，相当于在人体中放入了一个框架，“引导”患者自己的骨细胞沿着框架生长，当人体的骨细胞长成后，框架还可以在人体内自然消解。这种具有良好生物活性和强度的生物材料，将成为骨修复和替代的新材料，在修复或替代因外伤、病变等造成的骨组织局部缺损或坏死中，可通过植入生物材料辅助细胞和组织在其上再生。	http://cn.zglckf.com/Html/2009_09_26/2_65764_2009_09_26_66697.html
2009 年 8 月，美国犹他州大学研究人员研发出一种新型医用黏合剂，可以有效修复因车祸等原因造成的粉碎性骨折。研究人员是从一种名叫沙塔虫的海洋生物那里获得启发的。这种生物生活在海洋泥沙和礁石空隙里，能分泌出一种黏液，将沙子或贝壳黏在一起筑成巢穴并使其牢牢固定在礁石上。	http://cn.zglckf.com/Html/2009_10_30/2_65764_2009_10_30_80290.html
2009 年 8 月 5 日,日本媒体日前报道，日本早稻田大学和日本国防医学院的科学家研制出一种手术用“纳米薄膜”。它的厚度只有 75 纳米，是玻璃纸的千分之一。其可用于修补体内伤口，可在体内自行降解。研究人员称，这是世界上最薄的医用胶布。外科医生通常用缝合或钉合的方法处理伤口，有时他们也会采用覆盖纤维蛋白的医用薄膜，虽然纤维蛋白能使血液凝结，对伤口起到粘合作用，但它常发生与附近组织不必要的粘连。而这种透明的新型黏性薄膜采用一种从螃蟹壳中提取的物质和一种海藻胶质制成，具有极高的柔韧性，能在不用粘合剂的情况下黏附在器官之上，避免发生不必要的组织粘连。	http://cn.zglckf.com/Html/2009_11_27/2_65764_2009_11_27_83608.html
2009 年 7 月 23 日，在武汉召开的国家自然科学基金委化学科学部“十二五”分析化学学科发展战略研讨会上，中科院院士姚守拙提出，要警惕纳米材料对人体和环境的毒性作用，并建议尽早在国内开展关于纳米毒性的系统性研究。	http://cn.zglckf.com/Html/2009_10_20/2_65764_2009_10_20_69408.html