Xenographic bone graft materials safely prepared by compound surfactant*

Chen Lei, Sun Lei, Tao Jian-feng, Jiang Jian, Gao Xin-sheng, Jie Yong-sheng, Tian Wei

Abstract

BACKGROUND: Effect of acellular surfactant and biological safety of bone graft materials highly correlated with selection of surfactant; therefore, a novel compound surfactant was used to prepare acellular bone graft materials in this study.

OBJECTIVE: To evaluate acellular effect and biological safety of bio-derived bone tissue treated by a novel surfactant in order to obtain a safe and reliable bone graft material.

METHODS: Surfactant was prepared with anionic surfactant sodium dodecyl benzene sulfonate (ABS), anionic surfactant sodium fatty alcohol ether sulfate (AES) and distilled water at the ratio of 13:7:80. Fresh bovine cancellous bone and surfactant which was used to remove cells and lipid by two-step flow were used to prepare a novel bio-derived bone graft material. The histological and microscopic observations of microstructure were made. Also acute body toxicity test, hematolysis experiment, cell toxicity test and biological safety were assessed on surfactant-treated bio-derived bone graft material (STBB). A long-term animal experiment was conducted to observe the biocompatibility and biodegradability of STBB. The ultraviolet dispersion of light luminosity method was employed to measure the residual amount of surfactant in STBB.

RESULTS AND CONCLUSION: STBB was a whitish porous cancellous bone. No cell was found in bone lacuna, bone canaliculus was empty, and the collagen fiber had an order arrangement. Acute body toxicity test was qualified according to GB/T16886.11–1997 standard, hematolysis experiment was < 5%, and cell toxicity test was grade 0, confirming that STBB was safe. The remaining surfactant in STBB was lower than 0.1 g/L. The long-term animal experiment demonstrated that fiber was present at 4 weeks, bone lacuna had cellular growth and the fusion of STBB and host appeared. The STBB was partial absorbed by organism at 8 weeks and completely absorbed at 24 hours. The results indicated that STBB had an excellent biocompatibility and biodegradability. As a new bone implant material, STBB was safe and dependable for transplantation.

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INTRODUCTION

Xenographic bone graft materials especially for treating bone fracture or bone defect always cause delayed healing, disunion or even infection, which is mainly caused by the response of organism to materials. Therefore, graft materials with less immunogenicity are necessary for clinical application. Recent studies indicate that antigenicity of bone tissue mainly displays at surface of cells[1]. Previous studies also indicated that surfactant decreased antigenicity of xenographic bone tissue materials[2]. Therefore, surfactant was used in this study to remove cells from xenographic bone in order to prepare bio-derived bone graft materials with less antigenicity. Additionally, this study was designed to evaluate structure, biological safety, and residual amount of surfactant.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment at biological safety level.

Time and setting

This experiment was performed at Laboratory of Bone Tissue Engineering and Regeneration, Beijing Jishuitan Hospital between July 2005 and December 2006. Animal experiment was conducted at the Animal Laboratory of Beijing Jishuitan Hospital between September 2005 and December 2006.

Materials

A total of 30 New Zealand rabbits of half gender aging 8 months and weighing 2.0–2.5 kg were provided by Animal Laboratory of Beijing Research Institute of Trauma Orthopedics. All rabbits were randomly treated at 1, 4, 8, 12, 26, and 52 weeks following sacrifice, with 5 rabbits at each time point. Ten additional healthy mice aging four or five weeks and weighing 17–23 g were provided by Animal Laboratory of Beijing Research Institute of Trauma Orthopedics. All mice were randomly divided into experimental and control groups, with 5 mice in each group. The experimental animals were disposed according to ethical criteria.

Fresh bovine cancellous bone, sodium dodecyl benzene sulfonate (ABS), and sodium fatty alcohol ether sulfate (AES) were provided by Beijing Chemical Agent Company; numerical control ultrasonic cleaner (KQ-250DB) by Kunshan Ultrasonic Instrument Co., Ltd.; bone cutter (HBS-250) by Shenyang Fuji, Ind, Co., Ltd.; thermostat-controlled water-bath and spectrophotometer (UV-2401PC) by SHIMADZU; scanning electron microscopy (SEM-6380LV) by Electron Company, Japan; histotome (RM-2235) by LEICA; fibroblasts of L-929 mice by Beijing Research Institute of Trauma Orthopedics.

Methods

Preparation of ABS/AES

ABS solution was diluted in 1 mol/L NaOH (pH 6.5–7.5), and ABS, AES and distilled water were then mixed at the ratio of 13:7:80. The mixture was slowly shaken in water bath at 65 ℃ until the AES solution was completely dissolved. The prepared ABS/AES liquid was lemon yellow.

Preparation of bovine cancellated bone

Superior extremity of fresh bovine femur was obtained after removing surface fascia, connective tissue, and cortical bone. Cancellated bone was then sectioned into 3 mm × 3 mm× 3 mm sections, which were rinsed with water for three times, maintained in distilled water, and reserved in refrigerator at 4 ℃. On the next day, the sections were rinsed with distilled water for three times and reserved in triangle conical flask.

Preparation of STBB

40 g bovine sections were maintained in 1-L triangle conical flask and added with 400 mL ABS/AES solution based on the ratio of 1:10. The samples were ultrasound-shaken below 40 ℃ for 1 hour per time. The ABS/AES solution was changed 400 mL per time, and the samples were then ultrasound-shaken for 1 hour again. The operation was repeated until the samples were lemon yellow and clear. Next, the samples were ultrasound-rinsed with distilled water below 38 ℃, and the distilled water was changed every half hour, 400 mL per time, for 40 times in total. The last usage of distilled water was reserved. The STBB was finally prepared and freeze-dried.

Gross and scanning electron microscope observation

Appearance and microstructure were observed.

Histological observation

The prepared STBB was fixed in 10% volume fraction of neutral formaldehyde, decalcified with 30% formic acid, rinsed with distilled water again and again to remove rudimental formaldehyde and formic acid, paraffin-embedded routinely, sectioned into 3-μm sections, and stained with HE staining.

Residual volume of ABS/AES

ABS/AES was diluted in saline at concentrations of 1%, 0.1%, 0.01%, 0.001%, 0.000 1%, and 0.000 01%. Ultraviolet spectrophotometer was used to measure absorbance at 190–900 nm.

The absorbance of distilled water which was used to rinse STBB for the last time was detected at 900–190 nm. The measurement was compared with surfactant concentration gradient table to calculate residue quantity of ABS/AES.

Hemolysis rate

Hemolysis rate was calculated as the follows: hemolysis rate = (sample absorbance–negative control absorbance)/(positive absorbance–negative control absorbance) ×100%. Hemolysis rate was ≤ 5%, suggesting that the hemolysis test was qualified.

Hemolysis rate of STBB at various concentrations

Surfactant had the swelling effect on cells; therefore, hemolysis rate of surfactant at varying concentrations (1%, 0.1%, 0.01%, 0.001%, 0.000 1%, 0.000 01%, 0.000 001%, and 0.000 000 1%) was measured in order to avoid interference of residual surfactant. 0.2 mL diluted rabbit blood mixing with 10 mL saline was considered as negative control group, and 0.2 mL diluted rabbit blood mixing with 10 mL distilled water was considered as positive control group.

Biological safety of STBB

Hematolysis experiment, acute body toxicity test, cell toxicity test and long-term bone implantation test were conducted on STBB according to GB/T-16886 standard.

Hematolysis experiment

8 mL fresh anticoagulation rabbit blood was diluted in 10 mL saline to adjust the absorbance which was 0.8±0.3 at 545 nm when the 0.2 mL diluted rabbit blood was maintained in 10 mL distilled water. There were 3 test tubes in the experimental group, with 5 g bone material for each test tube. The bone materials were added with 10 mL saline. There were 3 test tubes in the negative control group, with 10 mL saline for each test tube. There were 3 test tubes in the positive control group, with 10 mL distilled water for each test tube. All the test tubes were maintained in water bath at 37 ℃ for 30 minutes, and 0.2 mL diluted rabbit blood was added in each test tube. Following mixing, the samples were maintained in water bath at 37 ℃ for 60 minutes and centrifuged at 750 *g* for 5 minutes. The supernatant was obtained to measure absorbance at 545 nm. The final absorbance was determined by average value of three parallel absorbances, and the mean absorbance was used to calculate hemolysis rate.

Acute body toxicity test

Six tubes containing 20 mL saline were divided into material group and blank group, with three tubes for each group. Every 4 g bone material was put in tube in the material group, and the samples were maintained in water bath at 37 ℃ for 120 hours. Next, the samples were mixed with the same leaching liquor and filtered with 4.5–9.0 μm aperture diameter funnel. The filtered solution was sterilized at 115 ℃, a standard atmospheric pressure, and (0.070±0.001) MPa for 0.5 hour. Five mice in the experimental group were treated with caudal intravenous injection of 50 mL/kg material leaching liquor, while 5 mice in the control group were treated with the same volume of blank leaching liquor. State, toxic appearance, number of died animal, and body mass of mice were detected and recorded at 24, 48, and 72 hours after injecting leaching liquor.

Cell toxicity test

24-well plates were divided into experimental group (*n*=10), negative control group (*n*=13), and positive control group (*n*=3). L-929 cells were inoculated at 4 × 10^4/well ad cultured in CO_2 incubator at 37 ℃ for 24 hours. Three plates in the negative control group were obtained to count cells, and liquid was changed in other plates. Fresh liquid was used in the negative control group, 64 g/L phenol culture liquid was used in the positive control group, and 50% fresh leaching liquor was used in the experimental group. All samples were cultured in CO_2 incubator at 37 ℃, and cells were counted at days 2, 4, and 7 so as to determine growth rate. Cells in the positive control group completely died, so growth rate could not be calculated. The relative growth rate was calculated in order to evaluate cell toxicity. The relative growth rate (%) = mean number of cells in the experimental group/mean number of cells in the negative control group × 100%.

Long-term bone implantation test

Fresh bovine cancellated bone was sectioned into 3 mm diameter and 6 mm length cylindrical rod. Following treating

with surfactant, the rod was sterilized with Cobalt-60. A total of 30 New Zealand rabbits were randomly divided into 6 groups according to sacrifice time of 1, 4, 8, 12, 26, and 52 weeks, with 5 rabbits for each group. All rabbits were treated with auricular vein intravenous injection of 0.1% pentobarbital (40 mg/kg), knee was lateral-cut, a round defect with 3 mm diameter and 6 mm depth was made at tibial plateau using low-speed electrodrill, and cylindrical bone materials were implanted. Following sacrificing, the samples were fixed with 10% formaldehyde, decalcified with 30% formic acid, paraffin-embedded, sectioned into 3 μm sections, and stained with HE staining. Histological evaluation was finally performed.

Design, enforcement, and evaluation

This study was designed by the first and second authors, conducted by the first, third, and fourth authors, and evaluated by the fifth, sixth, and seventh authors. All authors underwent professional training and this study was evaluated by blind method.

Statistical analysis

SPSS 12.0 software was used by the first author in this study. Measurement data were expressed as Mean ± SD and compared with *t* test. A *P*-value of < 0.05 was considered significantly.

RESULTS

Appearance and biological characteristics of STBB

STBB assumed a creamy white color, no gross impurities, a honeycomb structure, and hard consistence, while trabecular gap was obvious. Residue was not found in the aperture under microscope. The trabecular structure was complete, cell component vanished in the bone lacuna, bone canals were void and collagenous fibers were arranged in neat rows (Figure 1). STBB had a natural vesicular structure, the three-dimensional structure of bone tissue was retained, substantia spongiosa bone holes were mutually interlocked, and aperture was even-shaped (Figure 2). Average porosity was (75.4±7.6)%, pore diameter was (463.9±200) μm, the surface was clean, and no residue found.

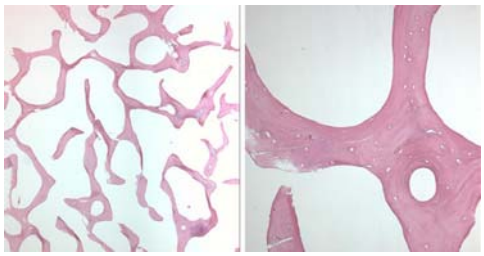

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Figure 1 The trabecular structure is complete, cell component vanishing in the bone lacuna, bone canals are void and collagenous fibers are arranged in neat rows

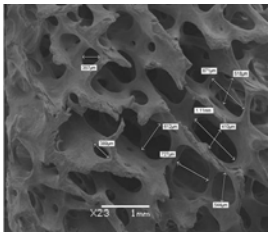
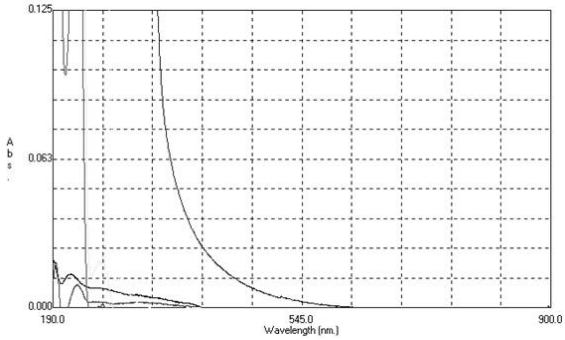


Figure 2 Surfactant-treated bio-derived bone graft material has a natural vesicular structure, the three-dimensional structure of bone tissue is retained, substantia spongiosa bone holes are mutually interlocked, aperture even-shaped, but varied-sized

Residue quantity of surfactant

Ultraviolet spectrophotometer was used to measure residue quantity in the distilled water which was used to rinse STBB for the last time at 190–900 nm. Absorption peak was found at 200–230 nm, which was calculated using surfactant concentration gradient table to obtain residue quantity of surfactant being lower than 0.001% (Figure 3).



ABS: anionic surfactant sodium dodecyl benzene sulfonate; AES: anionic surfactant sodium fatty alcohol ether sulfate

Figure 3 Measurement of residual quantity of ABS/AES in the bone material through the method of absorbance inference

Hemolysis rate of surfactant with various concentrations

Residue quantity of surfactant could not interfere hemolysis rate of bone matrix materials (Figure 4).

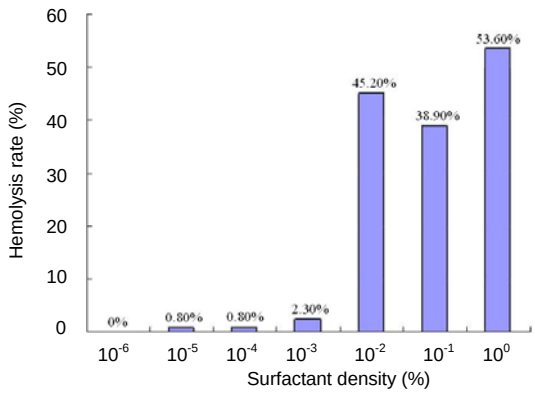


Figure 4 Hemolysis rate of surfactant at different concentrations

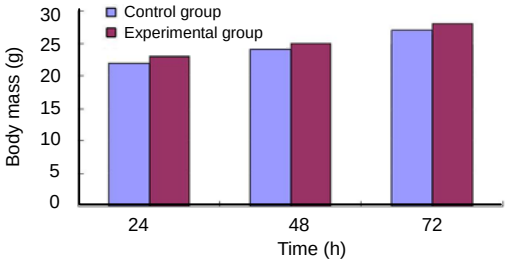
Biological safety evaluation

Hemolysis rate

The hemolysis rate of STBB was 3.53%, which was < 5% and considered as qualification according to GB/T16886.11–1997 standard.

Acute body toxicity test

Following an caudal intravenous injection of saline leaching liquor, mice in the experimental group did not have respiratory depression, breathlessness, movement reduction, cyanosis, ptosis, or even death. Additionally, examinations of heart, liver, kidney, and spleen were normal. Body mass was increased compared with control group (Figure 5). Therefore, the material was innocuous.



ABS: anionic surfactant sodium dodecyl benzene sulfonate; AES: anionic surfactant sodium fatty alcohol ether sulfate

Figure 5 Measurement of residual quantity of ABS/AES in the bone material through the method of absorbance inference

Cell toxicity test

Relative growth rate was 147.7%, 147.7%, and 136.9% at days 2, 4 and 7 respectively (Table 1). According to GB/T-16886 standard of relative growth rate (%) ≥ 100%, the cell toxicity was grade 0, *i.e.*, STBB did not have cell toxicity.

Table 1 Cell proliferation between material group and control group ($\bar{x}\pm s$)

Time	Negative control group ($\times 10^4$)	Material group ($\times 10^4$)	Relative growth rate (%)
Initial	4.0	4.0	–
24 h	6.0±0.3	6.0±0.3	–
2 d	9.0±0.6[a]	13.3±0.4	147%
4 d	44.0±4.2[a]	65.0±2.3	147.7%
7 d	14.6±1.2[a]	20.0±2.0	136.9%

[a]$P < 0.05$, *vs.* material group

Long-term bone implantation test

At 4 weeks, leucocyte was reduced, the counts of lymphocyte and plasma cell increased, production of textile fiber was obvious, cells were present in trabecular space, and STBB and host bone were in fusion (Figure 6). At 8 weeks, the leucocyte count continuously dropped, lymphocyte and plasma cell were present, textile fiber increased, and volume of STBB was smaller (Figure 7). At 12 weeks, a small number of leucocyte, lymphocyte and plasma cell were observed, textile fibers was massive, volume of STBB was smaller, and body was absorbed partially (Figure 8). At 26 weeks, residual STBB was observed, and they were irregular shapes and small volumes (Figure 9). At 52 weeks, HE staining revealed that no implants were visualized and STBB was absorbed completely (Figure 10).

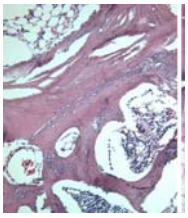


Figure 6 Leucocyte was reduced, the counts of lymphocyte and plasma cell increased, production of textile fiber was obvious, cells were present in trabecular space, and surfactant-treated bio-derived bone graft material and host bone were in fusion

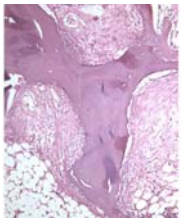


Figure 7 Leucocyte count continuously dropped, lymphocyte and plasma cell were present, textile fiber increased, and volume of surfactant-treated bio-derived bone graft material was smaller

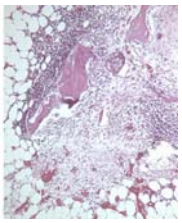


Figure 8 At 12 weeks, a small number of leucocyte, lymphocyte and plasma cell were observed, textile fibers was massive, volume of surfactant-treated bio-derived bone graft material was smaller, and body was absorbed partially

Figure 9 At 26 weeks, residual surfactant-treated bio-derived bone graft material was observed, and they were irregular shapes and small volumes

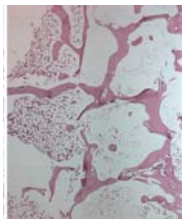


Figure 10 HE staining revealed that no implants were visualized and surfactant-treated bio-derived bone graft material was absorbed completely

DISCUSSION

Acellular method attracts more attention for preparing bone graft materials. Following acellular treatment, substances which may cause immunological rejection are removed from natural bone tissue[1], while initial structure and characteristics are reserved. Microenvironment of material surface is beneficial for adhesion and proliferation of cells[3-4].

Enzyme digestion, percolating solution, and surfactant cleaning are major acellular methods[5-6]. In particular, enzyme digestion is the strongest, but it can destroy cell matrix. Percolating solution is limited by some factors, while surfactant cleaning is a soft method. Surfactant has been widely reported to prepare cell stent materials. For example, sodium deoxycholate and sodium dodecylsulfate are used to prepare bio-valved stent[7-9]. However, nonionic surfactant TritonX-100 is widely used to prepare acellular matrix of bone tissue[5]. In order to choose an ideal defatting and acellular method, ABS and AES were selected in this study to prepare acellular bone matrix. Previous research[2] demonstrated that if the ratio among ABS, AES, and distilled water was 13:7:80, the surfactant liquid (ABS/AES) was the best for defatting and acellular effect.

It is important of biological safety for STBB considering as bone graft material. Results in this study demonstrated that residue of surfactant could not interfere hemolysis rate of bone matrix (Figure 10). Histological and electron microscope indicated that cell and fat were not found in bone trabecula, and cells in the bone lacuna disappeared completely, suggesting that acellular effect of surfactant was great. Long-term bone implantation test demonstrated that STBB had a well biocompatibility and biodegradation. Histological observation indicated that fibers were formed at 4 weeks, cells were observed in bone lacuna, and STBB and host bone were in fusion. At 8 weeks, fibers were increased, and peripheral bone tissue was absorbed by organism. Half year following implantation, the peripheral bone tissue was completely absorbed by the organism.

In a word, STBB has a well biocompatibility and biodegradation. Therefore, it is a safe and reliable material for bone implantation.

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用复合表面活性剂制备更安全的异种骨移植材料*

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摘要

背景：表面活性剂脱细胞的效果和骨移植材料的生物安全性与表面活性剂的选择有较大关系。因此，实验采用新型的复合表面活性剂制备脱细胞骨移植材料。

目的：应用新型表面活性剂处理生物源性骨组织，并进行脱细胞效果及生物安全性评价，以期得到一种更安全、可靠的骨植入材料。

方法：2 种阴离子表面活性剂十二烷基苯磺酸钠和脂肪醇聚氧乙烯醚硫酸钠以及蒸馏水以质量比 13∶7∶80 的比例配制复合表面活性剂。以新鲜的牛松质骨为原料，复合表面活性剂脱脂脱细胞两步法工艺，制备新型生物源性骨植入材料。

结果与结论：复合表面活性剂生物源性骨颜色呈乳白色，未见杂质，组织学及超微结构观察可见骨陷窝内细胞结构消失，骨小管空虚，胶原纤维排列整齐。生物安全性实验表明：按 GB/T16886.11-1997 标准急性全身毒性试验合格，溶血试验< 5%，细胞毒性试验 0 级。复合表面活性剂生物源性骨组织中表面活性剂的残留量低于 0.1 g/L。骨长期植入实验表明：植入材料与宿主骨融合良好，24 周后被机体完全吸收。说明复合表面活性剂处理的生物源性骨移植材料具有良好的生物相容性和生物降解性，是一种安全、可靠的骨植入材料。

关键词：脱细胞；表面活性剂；骨移植；骨移植材料

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