Osteogenic ability of cryopreserved bone marrow stromal cells complex *in vivo***☆

Zheng Yu-qian[1], Yuan Fang[2], Yan Fu-hua[1], Zhao Xin[1], Lin Min-kui[1]

Abstract

BACKGROUND: Our previous studies have demonstrated that cryopreserved bone marrow stromal cells (BMSCs) still maintain high survival rate, cell proliferation and osteogenic differentiation potentials after thawing. However, this result needs confirmed *in vivo* environment.

OBJECTIVE: To explore the effects of cryopreserved BMSCs and collagenic membrane BME-10X complex on type Ⅰ collagen synthesis *in vivo*.

METHODS: Beagle dog BMSCs were cultured *in vitro* and cryopreserved for 12 months, which were thawed and prepared complexes with collagenic membrane. The complexes were cultured with mineralization induction medium or normal medium for 5 days, followed by implanting into nude mice. The specimens were harvested and analyzed by gross observation, histopathological and immunohistochemistry at 4 weeks after implantation. The collagenic membrane cultured with mineralization induction medium served as controls.

RESULTS AND CONCLUSION: In the control group, the boundary of collagenic membrane was distinctly, without cell growth around boundary or intra collagenic membrane, additionally, there was little type Ⅰ collagen. In the non-induction group, cells grew into collagenic membrane, trabes-like collagen formed, and type Ⅰ collagen distribution increased at 4 weeks. In the induction group, scaffold degraded, more cells grew, and plenty of collagen formed osteoid-like tissues. The distribution of type Ⅰ collagen was obviously increased than that of other groups. The findings demonstrated that cryopreserved BMSCs possess strong osteogenic differentiation potentials after proliferation and induction combined with collagenic membranes *in vitro*.

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INTRODUCTION

As mesenchymal cells, differentiation potential of bone marrow stromal cells (BMSCs) has been confirmed by many studies, and has a wide application prospect in repair bone, cartilage tissue and periodontal tissue defects[1-3]. Our previous studies have confirmed that, medical collagenic membrane BME-10X can maintain and promote growth and amplification of BMSCs, which is an potential carrier in tissue-engineered field[4]. We also found that cryopreserved BMSCs still maintain high survival rate, cell proliferation and osteogenic differentiation potentials after thawing[5]. Based on previous studies, here, we aimed to construct cryopreserved BMSCs and collagenic membrane complex and to explore the effects of cryopreserved BMSCs and collagenic membrane BME-10X complex on type Ⅰ collagen synthesis in nude mice using immunohistochemistry, and to evaluate the osteogenic differentiation potentials of this complex.

MATERIALS AND METHODS

Materials

One healthy, adult, female, Beagle dog, weighing 11 kg, was provided by Shanghai Laboratory Animal Center of Chinese Academy of Sciences. Fourteen nude BALB/c mice, aged 4–6 weeks, weighing 18–23 g, were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences. Main reagents and instruments used in this study are as follows:

Reagent and instrument	Source
CO_2 incubator	Heraeus, Germany
Nitrogen canister	CBS, USA
Inverted phase contrast microscope	Olympus, Japan
New-born calf serum	Hangzhou Jiangbin Shengwu Keji Co., Ltd.,
DMEM	Gibco, USA
Metasolon, vitamin C, β-sodium glycerophosphate	Sigma, USA
Medical collagenic membrane BME-10X	Institute of Biomedical Engineering, Chinese Academy of Medical Sciences, China
Type I collagen antibody, SABC-peroxydase kit	Wuhan Boster Bio-engineering Co., Ltd., China
DAB reagent kit, EDTA antigen retrieval liquid	Fuzhou Maixin Company, China

Methods

Harvest of BMSCs

The BMSCs were obtained using “improved whole bone marrow culture”[6] and cryopreserved at the 2nd passage.

Cryopreservation and thawing of BMSCs

Cryoprotectant comprised 10% dimethyl sulphoxide, 10% new-born calf serum, 80% DMEM and pre-cooled. The 2nd passaged BMSCs were digested, centrifuged, and added into the cryoprotectant with final concentration of 2.5×10^9/L, at 4 ℃ 0.5 hour, –20 ℃ 0.5 hour and –80 ℃ overnight in a refrigerator and then placed in the liquid nitrogen. The cells were thawed at 37 ℃ thermostatic water bath, centrifuged, and preserved at standard environment for further use.

Induction of cryopreserved BMSCs

DMEM containing 10% new-born calf serum served as basic medium. Metasolon (10^{-8} mol/L), vitamin C (50 mg/L) and β-sodium glycerophosphate (10 mmol/L) were supplemented into basic medium as induction medium.

Preparation of BMSCs and collagenic membrane complexes

Medical collagenic membrane BME-10X was cut into 0.5 cm×0.5 cm pieces, exposed to viltalight lamp for 1 hour, and prewetted using basic or induction medium at 24-well plates. Cryopreserved BMSCs were passaged and incubated on prewetted collagenic membrane at final concentration of 1×10^7/L[7], with 30 μL cells in each piece, incubated in a 5% CO_2 incubator at 37 ℃ for 0.5 hours, followed by adding 2 mL basic or induction medium, which was changed every other day. Cell adhesion was observed under an inverted microscope. The cells were collected for further use at 5 days after *in vitro* culture[4].

Subcutaneously replantation of BMSCs and collagenic membrane complexes into nude mice

All 14 animals were randomly divided into 3 groups (Table 1). After ketamine anaesthesia (100 mg/kg), two 0.6-cm incisions were prepared at the back, exposed subcutaneous tissues, 1 piece of complex was implanted in each incision and sutured.

Table 1 Implantation of cell collagenic membrane complexes into nude mice and experimental grouping

Group	Complex	Nude mice (*n*)	Harvest time (wk)	Harvest number (*n*)
Induction	Collagenic membrane+ BMSCs+induction medium	5	4	5
Non-induction	Collagenic membrane+ BMSCs+basic medium	5	4	5
Control	Collagenic membrane+induction medium	4	4	4

Drawing materials

Activity, diet and wound of mice were observed after operation. All animals were sacrificed at 4 weeks after gross observation and tissues were fixed in 40 g/L paraform.

Histopathological examination

The specimens were demineralized in 10% EDTA, embed, and prepared 5 μm successive sections, followed by hematoxylin-eosin staining and observation under an optical microscope.

Immunohistochemistrical staining

The immunohistochemistrical staining was performed based on instruction of SABC-peroxydase kit. The coloration of collagen was brownish yellow. PBS (0.01 mol/L) substituted first antibody served as negative control.

Quantitative analysis method

The density of positive staining was represented by staining color, which was measured using Image-Pro Plus6.0 software. The immunohistochemistrical results were transformed into average absorbance selecting representative area from each section and measured at 4-high power fields. The stronger positive, the lower measured value[8].

Statistical analysis

All data were performed *t* -test using SPSS 12.0 software at α=0.05.

RESULTS

Gross observation

All animals acted normally, the skin of implanted region was integrated and smooth. No inflammatory reaction, graft prolapsed or immunological rejection. The harvested cell collagenic membrane complexes were harder than single collagenic membrane.

Histopathological examination

At 4 weeks after implantation, the boundary of collagenic membrane was distinctly, without cell growth around boundary or in internal collagenic membrane in the control group (Figure 1). There were cell grew at both sides and center of collagenic membrane in the non-induction group, collagen fiber degraded at one side, and tiny trabes-like collagen formed (Figure 2). In the induction group, cells grew into collagenic membrane at one side, collagen fiber degraded, and more cells grew, and tiny trabes-like collagen formed (Figure 3).

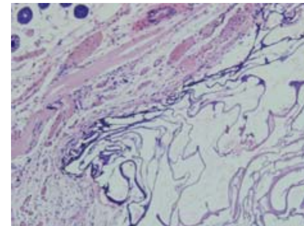

Figure 1 Histological findings of collagenic membrane in the control group at 4 wk after implantation, no collagenic membrane degraded (Hematoxylin-eosin staining, ×200)

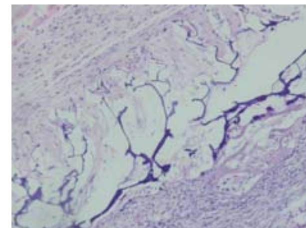

Figure 2 Histological findings of collagenic membrane in the non-induction group at 4 wk after implantation, collagenic membrane degraded and new collagen formed (Hematoxylin-eosin staining, ×200)

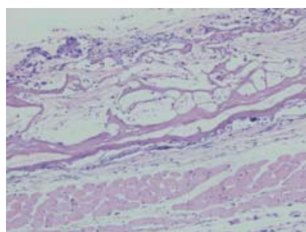

Figure 3 Histological findings of collagenic membrane in the induction group at 4 wk after implantation. Collagenic membrane degraded and plenty of new collagen formed (Hematoxylin-eosin staining, ×200)

Immunohistochemistrical results

There was no type Ⅰ collagen expression intra collagenic membrane, but few at collagenic membrane surface in the control group (Figure 4). There was type Ⅰ collagen expression in both the non-induction and induction groups. However, the type Ⅰ collagen expressed from collagenic membrane surface deep into intra collagenic membrane, which mainly concentrated at collagenic membrane surface and 1/3 of intra collagenic membrane in the non-induction group (Figure 5). Plenty of type Ⅰ collagen were expressed intra collagenic membrane in the induction group (Figure 6).

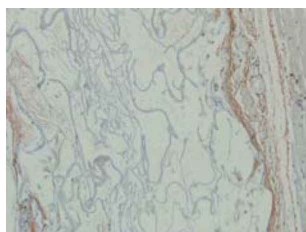

Figure 4 Type Ⅰ collagen expression in the control group at 4 wk after implantation (×200)

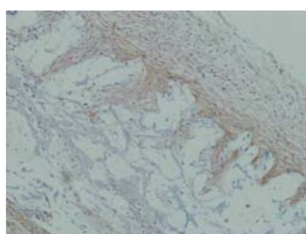

Figure 5 Type Ⅰ collagen expression in the non-induction group at 4 wk after implantation (×200)

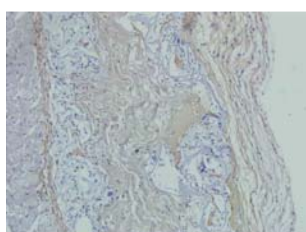

Figure 6 Type Ⅰ collagen expression in the induction group at 4 wk after implantation (×200)

Comparison of average absorbance value of type Ⅰ collagen expression between different groups

There were obviously differences among non-induction, induction and control groups in average absorbance value of type Ⅰ collagen expression, additionally, and the non-induction group was significantly different from the induction group (Table 2).

Table 2 Comparison of average absorbance value of type Ⅰ collagen expression in different groups ($\bar{x}\pm s$)

Group	Fields (*n*)	Average absorbance
Induction	20	0.196 3±0.012 6[ab]
Non-induction	20	0.148 9±0.003 7[a]
Control	16	0.077 5±0.005 8

[a]P < 0.01, *vs*. control group; [b]P < 0.01, *vs*. non-induction group

DISCUSSION

Bone tissue engineering is one of the most potential fields to achieve actual benefits. BMSCs are characterized by wide sources, simple harvest, and small damage to patients, thus, they are hopeful to utilize in clinic. Plenty studies have demonstrated that BMSCs can differentiate to osteoblasts and have extensive application prospects in repairing bone tissue, soft tissue or periodontal tissue defects[1-3].

With penetration and development of cell biology and tissue engineering, more BMSCs are needed, accordingly, how to preserve cells long term and massively are necessary. Our previous studies have confirmed that, cryopreserved BMSCs still maintain high survival rate, cell proliferation and osteogenic differentiation potentials after thawing[5]. However, these results need to be confirmed *in vivo* environment. Medical collagenic membrane is benefit for amplification of BMSCs and periodontal ligament cells[4,7,9]. Previous researched also found that cells grew faster on cell-scaffold compound culture, and cell population reached a stabilization at 5 days after culture[4]. The cells achieve maximum growth and proliferative activity at 5 days *in vitro* culture, so it has great significance to explore the growth and osteogenic capability i*n vivo*.

Type Ⅰ collagen is the most abundant collagen of the human body, protein of which occupies 80%–90% of organic principle. It has importance in maintaining bone structure integrity and biomechanical property[10-12]. Type Ⅰ collagen forms protein framework of bone matrix, its synthesis and secretion is the precondition for bone tissue formation. In addition, the ordered arrangement of type Ⅰ collagen is the main factor in forming mechanical force of bone tissues[11-12]. Osteoblasts secrete massive type Ⅰ collagen during osteogenesis, consequently, metabolic parameters of type I collagen can reflex bone metabolic levels, which is superior to former biochemical indicators in sensitivity, specificity and stability[12]. Accordingly, type Ⅰ collagen expression was observed to explore its effect on osteogenic capability.

Here, we found that the cryopreserved BMSCs grew well on collagenic membrane after thawing; the Collagenic membrane degraded and plenty of new collagen formed at 4 weeks after complex implantation into nude mice, however, these changes

could not been seen in the control group. Moreover, there were more type Ⅰ collagen expression in the induction group than that of the non-induction group, suggesting mineralization solution promotes BMSCs *in vitro* osteoblast differentiation and *in vivo* osteogenic differentiation. The degradation, cell number and type I collagen expression presented with asymmetry at both sides of collagenic membrane, maybe results from one side culture of BMSCs. This finding revealed that cells should be incubated multidimensional on the scaffold in order to fasten proliferation amount and elevate osteogenic differentiation in unit time when reconstruction bone utilizing tissue engineering technique.

The findings demonstrated that cryopreserved BMSCs not only maintain high survival rate, cell proliferation and osteogenic differentiation potentials after thawing[5], but also have strong osteogenic differentiation combined with collagenic membranes *in vitro*. These findings provide theoretical basis for tissue engineering repair using cells from a same origin.

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冻存骨髓基质细胞复合材料体内成骨基质的合成能力**☆

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摘要

背景：课题组以往研究显示：体外培养条件下，冻存骨髓基质细胞复苏后仍保持较高的细胞存活率、细胞增殖及成骨分化能力。上述结果仍然需要进一步在体内环境下证实。

目的：观察经超低温冻存后的骨髓基质细胞和支架材料胶原膜 BME-10X 复合体植入裸鼠体内后 I 型胶原的合成情况。

方法：体外分离培养 Beagle 犬骨髓基质细胞，冻存 12 个月后复苏，体外构建骨髓基质细胞和胶原膜材料复合体。分别经矿化诱导培养液、基础培养液培养 5 d 后，植入裸鼠体内，于术后第 4 周取出标本，进行大体观察、组织病理学和免疫组化分析，并应用图像分析系统对各组标本中的 I 型胶原进行定量分析。以矿化诱导培养液培养的单纯胶原膜材料为对照组。

结果与结论：对照组在植入胶原膜后，胶原膜边界清晰，膜边缘及内部基本没有细胞生长，Ⅰ型胶原分布很少；在未诱导矿化组，术后第 4 周可见，胶原膜内有细胞长入，并有细小的条索状新生胶原形成，Ⅰ型胶原分布明显增多；在诱导矿化组，植入后也可见支架材料的分解降解和更多的细胞生长，大量新生的胶原形成类骨质样组织，与前两组对比，Ⅰ型胶原分布增多有显著性意义。结果表明冻存骨髓基质细胞复苏后进行体外培养扩增与诱导分化，并在体内环境下复合胶原支架材料，仍然具有较强成骨能力。

关键词：骨髓基质细胞；胶原膜；免疫组化；裸鼠；成骨能力

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