Morphological study of dynamic culture of thermosensitive collagen hydrogel in constructing tissue engineering complex

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ABSTRACT
The purpose of this study is to research the morphologies and functional characteristics of the cell-scaffold complex in vitro constructed under dynamic culture conditions. BMSCs were isolated from the long bones of Fischer344 rats, and performed in vitro amplification to the third generation as seed cells, together with thermosensitive collagen hydrogel (TCH) as cell adhesion matrix, and poly-L-lactic acid (PLLA) as scaffold, to construct cell-scaffold complex. The cell-scaffold complexes in the experiment group and the control group were then performed dynamic culture and static culture. After 7 d of in vitro culture, the complexes in the 2 groups were performed gross observation and SEM; meanwhile, the total DNA content in the complex was detected on D0,1,3, and 7 of culture. After cultured using these 2 ways, collagen could both wrap the PLLA scaffold, forming dense film-like structures on the PLLA surface. The total DNA contents in the cell-scaffold complex of the experiment group on D1,3, and 7 were significantly higher than the control group (P < 0.05). Compared with D0, the total DNA contents on D1,3, and 7 in both groups were gradually increased, but only the total DNA contents on D7 showed statistically significant difference than D0 (P < 0.05). TCH -PLLA fiber joint-constructed complex extracellular matrix had good biocompatibility, and dynamic culture could promote the distribution of BMSCs on the surface and inside the structure, thus promoting cell proliferation, so it could be used for the in vitro construction of tissue engineering complex.

Introduction
In vitro construction of cell-scaffold complex using tissue engineering methods for repairing tissue and organ defects has become one hot research field of regenerative medicine currently, but problems, such as cells in in vitro constructed complexes were insufficient, unevenly distributed, and could not express their biological activities well, have not been effectively solved. In vitro construction using dynamic culture methods, such as bioreactor, could enhance cells’ biological activities, and improve the functional characteristics of the complex, noted by Liu M et al. Single-component extracellular matrix scaffold could not meet the in vitro construction requirements of tissue engineering, and the combination of a variety of natural and synthesized biologically active materials with good biocompatibility have exhibited good research and application prospects, noted by Cen L et al.

This study used BMSCs as seed cells and PLLA fiber woven material as extracellular matrix scaffold to construct mesh spatial structure, due to the multiple desirable characteristics of PLLA including renewability, biocompatibility, transparency, and thermoplasticity, noted by Lin Xiao et al., and then used TCH as cell adhesion matrix to build tissue engineering complex under dynamic culture conditions, aiming to observe the complex morphological characteristics and to test the proliferation status of the seed cells, thus providing reference for the in vitro construction of tissue engineering complex.

Materials and methods
Animals, reagents, and instruments
10 7-week-old male Fischer 344 rats, SPF grade, average body weight 250g, were provided by the
Experimental Animal Center of Jilin University. The TCH culture kit (Nitta-gelatin, Japan) was composed of swine-derived collagen type I, non-NaHCO3-containing 10-fold concentrated dulbecco’s modified eagle medium (DMEM) and buffer (260 mmol/L NaHCO3, 50 mmol/L NaOH, 200 mmol/L glycolate piperazine ethanesulfonic acid), which could keep dynamic flow status below 10°C while gelled at 37°C.

The PLLA scaffold was woven from 10 mm in-diameter PLLA fibers (Fig. 1, stained with trypan blue, bar is 1.0 mm), the scaffolds diameter was 3.0 mm, the breaking strength was 366 N when pulled with a speed as 100 mm/min, and the material was hydrophilically prepared using 70% ethanol and DMEM culture medium before usage. DNA extraction kit (Wako, Japan); RNA extraction kit (Wako, Japan).

**Isolation, cultivation, and identification of BMSCs**

The Fischer344 rats were anesthetized using under overdose ether, and then isolated bilateral tibial and femoral bones under sterile conditions; the bone ends were then removed, and the marrow cavity was then washed using 5 mL syringe equipped with DMEM culture medium; the bone marrow was then centrifuged (radius 10 cm) at 4°C and 1500 r/min for 10 min; after the supernatant was discarded, the precipitate was re-suspended using DMEM and then placed into T-75 flasks for culture using 10% fetal bovine serum (FBS), 100U/mL penicillin, and 100 mg/mL streptomycin-containing DMEM at 37°C, 5% CO2, and saturated humidity. The medium was exchanged for the first time 4 d later, and when the cells grew to 80% fusion, 0.25% trypsin – 0.02% EDTA was added in for the digestion; the cells were then performed amplification and passage with the ratio as 1: 2, noted by Zhang CS et al. The third generation cells were then performed osteogenic and adipogenic differentiation, and identified as BMSCs, noted by Gambardella A et al.5-9

**Grouping and methods**

The TCH stock solution was diluted using DMEM culture medium with the ratio as 9: 1 to prepare TCH cell culture solution, which was then added BMSCs to prepare the cell suspension; the BMSCs cell suspension (1 × 106 cells/mL) was then seeded onto the PLLA scaffold, and the cell-scaffold complex was then placed in one 6 mm in- bottom diameter and 10 mm in -height cylindrical culture vessel (in order to prevent cell loss) for 30 min pre-incubation at 37°C, 5% CO2, and saturated humidity (so that the collagen was kept in gel status); the cell-scaffold complex was then transferred into a dynamic culture device as the experiment group. The dynamic culture device was specially designed to make DMEM flow back and forth, swing angle 70° (35° on superior and inferior horizontal plane, respectively) and swing frequency 0.5 Hz; the cell-scaffold complex could scroll back and forth in the flask with the culture medium, and the medium was changed every day. Additionally, another cell-scaffold complex was cultured using ordinary stationary plate at 37°C, 5% CO2, and saturated humidity as the control group.

**Observation indexes**

**Gross observation, observation light microscope and SEM**

After in vitro cultured for 7d, the cell-scaffold complexes of the 2 groups were performed gross observation. Then, they were cut along the vertical or horizontal axis, washed with PBS, dried at critical points, and gold sputtering for morphological observation of the surface, cross section, and longitudinal section by light microscope and SEM, noted by Scott JB et al.10
**Total DNA quantification of the cell-scaffold complex**

The cell-scaffold complexes of the 2 groups were randomly selected after *in vitro* cultured for 0, 1, 3, and 7 d (n = 5), and quantitatively detected and analyzed the total DNA content using DNA/RNA extraction kit in accordance with the kit instructions: firstly, the complex was cut into 1 mm × 1 mm × 1 mm pieces, treated with an ultrasonic cell crusher and then centrifuged (radius 10 cm) at 1000 r/min for 5 min; the supernatant was then collected and detected the absorbance at 260 nm (a) using one UV spectrophotometer; the simple 3rd generation BMSCs were used to prepare the DNA concentration standard curve, noted by Lundborg G et al.11

**Statistical methods**

SPSS17.0 statistical software was used for the analysis, and the data were expressed as mean ± standard deviation; the intragroup comparison before and after surgery used ANOVA, and the pairwise comparison used the SNK test; the intergroup comparison used the independent sample t test, with α = 0.05 as significance level.

**Results**

**Gross observation, observation light microscope and SEM**

After two culture ways, only the experimental group could be observed TCH wrap the PLLA scaffold (Fig. 2). Observation light microscope showed in and around these 2 types of the cell-scaffold complex the cells arranged closely embrace the PLLA scaffold (Fig. 3). SEM showed the surface of the 2 types of the cell-scaffold complex formed one multi-layer cells-constructed film-like structures (Fig. 4); the cells on the complex surface of the experiment group were spindle and arranged neatly along the long axis, with good cell activities and villose preiection visible on the surface (Fig. 5); the cells on the complex surface of the control group were extended and flat (Fig. 6). The longitudinal section of the experiment group showed plenty of cells filling the PLLA fibers (Fig. 7), but the adherent cells of the control group were less (Fig. 8). the cross sections of the experiment group and the control group showed a lot of collagen fibers filling the PLLA scaffold, forming porous 3-dimensional

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**Figure 2.** Observation light microscope of BMSCs-ECM scaffold composites in 2 groups after 7 d of culture. Vertical section observation in the experimental group (×40) Black arrow indicated collagen fiber, white arrow indicated PLLA fiber.

**Figure 3.** Cross section observation in the experimental group. Black arrow indicated PLLA fiber, white arrow indicated cells.
Figure 4. SEM observation of BMSCs-ECM scaffold composites in 2 groups after 7 d of culture. Surface observation in the experimental group (×300) Black arrow indicated the membranous structure, white arrow indicated PLLA fiber.

Figure 5. Surface observation in the experimental group (×1000).

Figure 6. Surface observation in the control group (×1000).

Figure 7. Vertical section observation in the experimental group (×600) Black arrow indicated PLLA fiber, white arrow indicated cells.
structures (Fig. 9), and there was no significant difference between them.

**Total DNA content analysis on cell-scaffold complex**

In addition to D0, the total DNA contents on the experimental cell-scaffold complex on *in vitro* culture D1, 3, and 7 were statistically significantly higher than the control group (P < 0.05). Compared with the content on D0, the total DNA contents of the 2 group on D1, 3, and 7 were gradually increased, but only the total DNA contents on D7 showed statistically significant difference than those on D0 (P < 0.05). Table 1.

**Discussion**

In recent years, tissue engineering *in vitro* culture uses seed cells and biological materials to build tissue engineering complex for repairing tissue defects, and has become one hot research of regenerative medicine, noted by Konofaos P et al.12 Researchers believed the prerequisites of tissue engineering regeneration to be the cells with certain differentiation capacities, biodegradable scaffolds, and various cytokines, noted by Marquard LM et al.13-16 This study used classical method of tissue engineering technology, using BMSCs, which had multi-directional differentiation potentials, as seed cells, and TCH-PLLA fiber co-produced complex scaffold as extracellular matrix to construct tissue-engineering complex in a dynamic culture environment, and then preliminarily studied its morphologies and cell proliferation characteristics. TCH was mainly composed of collagen type I, and as a natural material, it had large advantages in tissue engineering research. The combination of TCH with PLLA or other biodegradable materials such as the poly(propylene fumarate)/calcium sulfate/β-tricalcium phosphate (PPF/(CaSO4/β-TCP)) composite, which could be degraded in vivo or vitro, noted by Cai ZY et al.,17,18 could obtain extracellular matrix complex with both good biocompatibility and certain mechanical strength, noted by Feinberg AW et al.19-21 Type I collagen is one of the most widely distributed and most important proteins in human body, and its porous structures and biological characteristics of retaining large amounts of water could help the metabolism of the cells in the complex, noted by Johnson EO et al.22,23 This study used TCH hydrogel
matrix as cell adhesion so as to effectively maintain the number of the seeded cells, thus restricting the cell mobility to some extent and reducing the loss of cells in the early seeding stage, and the stent material could have sufficient number and good distribution of original cells, and reduce the crawling process that the cells needed to enter the porous materials. Certain studies had shown the importance of a number of cellular components in in vitro constructing tissue engineering complex, noted by Liu H et al.5,24,25 During the culture process of the complex, the complex would gradually shrink, so the intercellular space would be denser, and this feature might be related with collagen components and a certain number of cells contained inside, and some study suggested that the cellular components inside would promote the culture-shrinkage characteristics of the complex, noted by Ding K et al.21,26 be beneficial for the interactions among cells, play organization coordination functions, and construct the organization with certain functional activities.

Bioreactors could effectively promote the in vitro growth of 3D complex structure, and promote the cells to express their biological activities, noted by Sun T et al.27-29 Dynamic culture device-generated hydrodynamic stimuli could provide a better cell-living environment, promote seed cells to adhere to the scaffold surface, and enhance the biological activities of the cells, noted by Sun T et al.27 The results of this study showed that compared with the static culture, the cells cultured under dynamic conditions showed better cell adhesion and growth, and the total DNA content was increased significantly. In constructing complex, the cell arrangement has very important significance for the biological behaviors of the cells. Certain nerve tissue regeneration researches found that during the process of axonal regeneration, the nerve cells arranged spontaneously parallel to Schwann cells in the injured areas of peripheral nerve and central nerve, noted by Guenard V et al.30,31 and during graft and reconstruction, regularly arranged cells could help to promote tissue formation and tissue regeneration, noted by Lietz M et al.32,33 thus providing certain advantages for tissue cells in terms of axial growth, noted by Miller C et al.32,34 SEM of this study found that BMSCs on the 7th day of dynamic culture were arranged parallel on the complex surface, and formed one film-like structure on the surface, thus wrapping the seed cells and biodegradable materials into a relatively stable environment, and maintaining the exchange of nutrition materials; these features could ensure the purity and quantity of the seed cells after transplantation, and lay foundation for organizational re-construction. These organizational features might have important implications for tissue engineering studies targeting peripheral nerves and tendons.

### Conclusion

The combination of biodegradable materials and collagen could construct extracellular matrix with porous 3-dimensional structures, and thus effectively improving the cell compatibilities of the materials; pre-incubation could limit the cell mobility to a certain extent, reduce cell loss in the primary construction stage, improve the grafting efficiency of seed cells, and improve the cell distribution among the 3-dimensional porous materials. Dynamic culture could provide the cells with hydrodynamic stimuli, thus improving the arrangement and distribution of the cells on the material surface and inside, increasing the cell activities, and promoting the proliferation of seed cells, so it could better meet the most fundamental requirements of in vitro constructing tissue engineering complexes.

This study focused on in vitro constructing tissue engineering complexes, did not research the specific aspect of complex organizations, and only

### Table 1. The comparison of total DNA content in 2 groups of cells – scaffolds complex in vitro at each time point. (n =5, x ± s).

| Group          | 0 d     | 1 d     | 3 d     | 7 d     | Statistic  |
|----------------|---------|---------|---------|---------|------------|
| Experimental   | 7.50 ± 1.01 | 13.27 ± 1.13 | 19.91 ± 3.05 | 22.57 ± 3.44* | F = 31.440 |
| Control        | 7.50 ± 1.01 | 8.66 ± 1.33  | 10.49 ± 1.60  | 11.77 ± 0.85* | P = 0.000  |
| Statistic      | t = 0.000 | t = 5.293 | t = 5.475 | t = 6.090 | P = 0.002  |

*Compared with preoperative value, P < 0.05
performed induction toward the seed cells’ pluripotent differentiation potentials instead of further studying the biological activities of the cellular components in the complex. This tissue engineering complex construction mode could be applied to a variety of tissue engineering researches, thus providing reference for in vitro constructions of such tissue engineering complexes as peripheral nerve regeneration and tendon tissue engineering.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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