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学位論文題目	*In Situ* Collagen Gelation:A New Method for Constructing Large Tissue in Rotary Culture Vessels （*In situ*におけるコラーゲンのゼラチン化法：回転式培養装置による新しい組織構築法）
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別紙様式３

論 文 内 容 要 旨

※整理番号	478	氏 名（しめいふりがな）	ソ ナンショウ 蘇 南彰
学位論文題目	*In Situ* Collagen Gelation: A New Method for Constructing Large Tissue in Rotary Culture Vessels (In situにおけるコラーゲンのゼラチン化法：回転式培養装置による新しい組織構築法)		

Purposes

1. To test the feasibility of application of *in situ* collagen gelation in rotary 3-D bioreactors.
2. To challenge the size of existing cultured tissue.
3. To establish a method for future bone tissue engineering.

Materials and Methods

Isolation and culture of newborn rat calvarial cells

Calvarial cell populations were isolated from the calvaria of 20 Wistar rats (2 days old) by collagenase sequential digestion.

Application of in situ collagen gel in high-aspect-ratio rotary vessels (HARVs)

Rotary bioreactors used in this study were HARVs. Culture space was about 8 cm in diameter × 1 cm in height.

1. Solutions for each vessel.

Solution A: 20 ml of bovine collagen solution (type I-AC; 0.5%, pH 3) + 10 mg of polymerized hyaluronic acid (HA).
Solution B (constitution buffer): 2.5 ml mixture of 50mM NaOH, 260mM $NaHCO_3$, and 200mM HEPES
Solution C (constitution medium): 1.25 ml fetal bovine serum (FBS) + 1.25ml 10x concentrated Eagle's MEM + 100 nM dexamethasone + 5 mM beta-glycerophosphate.
Solution A, B, and C were stored at 4℃.
Solution D: 2.5×10^6 rat calvarial cells in 0.1 ml culture medium.
Culture medium: alpha-MEM + 15 % FBS + 1% L-alanyl-L-glutamine + 50 μg/ml penicillin + 50 μg/ml streptomycin + 100 μg/ml neomycin + 5 mM beta-glycerophosphate

2. Mixing and 3-D gel constitution.

Solution A, B, C, and D were mixed in order at 4℃. The oxygenation membrane of the rotary vessel was first wetted with 0.5 ml FBS to avoid the adherence of gelated collagen to it. The cell-collagen mixture was injected into the rotary vessel and allowed to gelate in an incubator for 30 min at 37℃ in 95% humidified air/ 5% CO_2. Initial rotation speed was set at 15 rpm. Medium was changed every 2 days. Culture periods were 1, 2, or 3 weeks.

Analyses

1. Evaluation of the shrinkage of collagen gels in rotary vessels.
2. Microscopic observation: Differential interference contrast microscopy (DIC) and confocal laser scanning microscopy (CLSM; SYTO 10/ethidium homodimer-2 fluorescent staining) + scanning electromicroscopy (SEM).
3. Evaluation of metabolic activity: Alkaline phosphatase assay and MTT assay.
4. Evaluation of cell viability: Trypan blue exclusion and flow cytometry.
5. Statical data were shown as the mean±standard deviation (S.D.) and compared with Student's *t*-test.

（備考）１．論文内容要旨は、研究の目的・方法・結果・考察・結論の順に記載し、２千字程度でタイプ等で印字すること。
２．※印の欄には記入しないこと。

Results

Collagen gels contracted and became denser over time. The contraction was prominent within two weeks. The size of collagen gels decreased from 4 cm in diameter × 0.5 cm in thickness (week 0) to about 2.8 cm in diameter × 0.25 cm in thickness (week 3).

Living cells were homogeneously distributed in the collagen gels. Rat calvarial cells flattened and protruded processes to make contact with neighboring cells. Collagen interstices were smaller than 10 μm and many lacuna-like pores (about 10-20 μm in diameter) were found. Microvilli were more abundant on the cell surface of 3-week old samples. Calcified deposits were found on the cell surface and on collagen fibers of 2 and 3-week old samples. Opacity of the samples was also observed in the 2 and 3-week old samples.

Alkaline phosphatase content increased to 195% and MTT metabolites increased to 113% from week 1 to week 3. Trypan blue exclusion and flow cytometry analysis showed a corresponding decrease of cell viability. The cell viability of 3-week old samples in trypan blue exclusion and flow cytometry analysis were 89.9±1.9% (n = 5) and 90.5±1.0% (n = 5), respectively.

Discussion

To our knowledge, this is the first investigation using *in situ* collagen gelation in rotating bioreactors for the purpose of tissue engineering, and we have produced the largest dense tissue engineered *in vitro* (~ 2.8 cm in diameter × 0.25 cm in thickness). *In situ* collagen gelation is a new method of applying the static 3-D collagen culture method to high-aspect-ratio-vessel (HARV) rotating bioreactors. This method supports a growing dense tissue in vitro with close to 90% viability for at least 3 weeks and is therefore suitable for large tissue engineering *in vitro*.

Calcification was found in most 2-week old samples and all 3-week old samples. Despite of the effect of calcification, cell viability retained about 90% after 3 weeks. Since razor-cutting without immediate tissue fixation may cause cell death at cutting surface, cell viability should have been slightly higher than the data in this study.

Scaffold macroporosity has been considered to be a critical factor in cell migration, matrix deposition, and mineralization for bone tissue engineering. Pore sizes larger than 100-200 μm have also been considered necessary for bone formation *in vitro*. However, our results showed that the interstices of the *in situ* collagen gel were smaller than 10 μm. Despite small porosity, cells were viable and migrated in the collagen gel, and formed lacuna-like cavities (~20 μm in diameter). Hence, our results seemed to conflict with the rule of scaffold macroporosity. The reason might be due to different methods of cell seeding: traditional methods seeded cells by immersing scaffolds in cell suspension but our method, the *in situ* gelation, seeded cells by embedding cells homogeneously in the collagen gel. In fact, in the cortical bone, Haversian canals were 22-110 μm in diameter and lacunae were about 10 μm in diameter. Our method seems to be morphologically similar to the bone tissue.

Conclusion

1. Based on the evidence of high cell viability and homogeneous cell distribution, *in situ* collagen gelation in rotary 3-D bioreactors is a feasible method for *in vitro* tissue engineering.
2. A large dense tissue (~ 2.8 cm in diameter × 0.25 cm in thickness) was obtained in this study. We believe that the thickness of cultured tissue can be further challenged, if the height of the culture space can be increased.
3. Despite of the effect of calcification, cell viability retained high in this study. We consider that *in situ* collagen gelation in rotary bioreactors might be applied to bone tissue engineering.

(987 words)

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学位論文審査の結果の要旨

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（学位論文審査の結果の要旨）

酸素透過膜と培養液入出口のついた培養容器を持つ回転式培養装置（Wolf and Schwarz, 1991）において、培養細胞の足場としてビーズのような小さなものしか使用されていない。本研究はこの回転式培養装置とin situ コラーゲンゼラチン化法を組み合わせ、体外骨組織の作成を試みた。

新生仔ラットより頭蓋骨細胞を単離し、コラーゲン溶液（４℃）と混和し、培養容器（直径 ８㎝、高さ１㎝）に入れた。３７℃で静置し、コラーゲンをゼラチン化した（直径 ８㎝、高さ0.5 ㎝）。さらに容器を培養液で満たし回転させた（15 rpm）。培養液は48時間毎に交換した。

培養３週後において、コラーゲンゲルは直径2.8cm、高さ0.25cmであった。コラーゲンゲルの中に、生きた細胞は均等に分布していた。細胞の生存率は90％であり、代謝活性はわずかに増加していた。骨細胞の形態を示し石灰沈着も見られた。

本研究は新しい培養方法を開発し、報告のある中では最大の体外骨組織の作成に成功した。今後の再生医学の研究に寄与するものであり、博士（医学）の授与に値する。

（平成16年　2月20日）