Letter to the editor

Effect of cell seeding methods on the distribution of cells into the gelatin hydrogel nonwoven fabric

1. Introduction

Recently, as one trial to enhance the biological functions of cells, several attempts have been performed to allow cells to interact in a three dimension (3D) manner. To do that, it is of prime importance to achieve a homogeneous distribution and proliferation of cells in 3D scaffolds, which affects the biological functions of cells [1]. The researches of 3D cell culture scaffolds have been extensively reported while several materials with different inner structures have been prepared from synthetic polymers, natural polymers, and bioceramics [2].

There have been reported on 3D scaffolds with various inner structures [3], such as sponge [4], 3D printer structure [5], fiber (including nanofiber) [6], woven fabric [7], non-woven fabric [8], and a braid [9]. Among them the effect of seeding methods on the cell distribution has been evaluated to hydrogel scaffolds [10,11]. For the hydrogel scaffolds reported so far, the mechanical property is too poor to allow cells to seed homogeneously in the scaffolds [12]. As a trial to break through the issue, the hydrogel of gelatin non-woven fabrics has been explored to demonstrate a homogeneous proliferation of cells in the non-woven fabric [13].

This study is undertaken to evaluate the effect of cell seeding methods on the proliferation and distribution of cells in the gelatin hydrogel non-woven fabrics (GHNF). We examine the proliferation behavior of cells and their membrane damage based on lactate dehydrogenase (LDH) activity after different cell seedings.

2. Materials and methods

2.1. Preparation of GHNF

The gelatin hydrogel nonwoven fabrics (GHNF) were prepared by a solution blow spinning method reported by Nakamura et al. [13,14]. The spinning was performed at an air pressure of 0.275 MPa and the working distance of 50 cm with the syringe pump nozzle of inner diameter 250 μm. Then, the GHNF were freeze-dried for 72 h. The non-crosslinked and dried GHNF were treated in a vacuum oven at 140 °C and 1×10^{-5} MPa for the dehydrothermal crosslinking of gelatin for 48 h according to the method previously reported [15]. The cross-linked GHNF were punched out into 4-mm-diameter dried disks (the weight and thickness of each disc were 4.06 ± 1.27 mg and 0.74 ± 0.04 mm), and then the disks were placed into each well of 48 multi-well culture plate (Costar;

Corning Life Sciences, New York, USA), followed by sterilization of ethylene oxide gas.

2.2. Cell culture experiments

KUM6 cells of a mouse bone marrow-derived mesenchymal stem cell line were purchased from JCRB Cell Bank (National Institute of Biomedical Innovation, Health and Nutrition, Osaka, Japan). The cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, GIBCO Life technologies Co., Carlsbad, CA, USA) containing 10% bovine fetal calf serum (FCS, Hyclone laboratories, Inc., Utah, UT, USA) and 1% penicillin and streptomycin at 37 °C in a 5% CO_2-95% air atmospheric condition. The cells were detached with 0.25% trypsin-containing 1 mM EDTA solution (Nacalai Tesque. Inc., Kyoto, Japan), and continued to culture in 100 mm cell culture dish (Corning Life Sciences, New York, USA) to allow to grow until to 80% confluency.

2.3. Specification of swelling rate of GHNF

The dried GHNF weight was measured and swollen in DDW. The weight after swelling was measured, and the amount of water (mg) sorbed by the GHNF was calculated. Theoretically, water at 10 times of GHNF weight is absorbed in the GHNF. In the state where 10 times weight medium was absorbed, the value was defined as 100%. For example, 5 mg of dried GHNF can absorb 50 μg (μl) of water.

2.4. Cell seeding and culture

The cells were seeded into GHNF by the two seeding methods of a pre-swollen and dried seeding at a cell density of 1×10^5 cells/scaffold. As the pre-swollen seeding, after swelling by the medium for 30 min, the excessive medium was excluded from the pre-swollen GHNF by a pipette aspiration. The swelling rate of GHNF was about 37%. Then, the cell suspension (2×10^6 cells/ml, 50 μl) was added on the top of GHNF. As the dried seeding, the cell suspension (2×10^6 cells/ml, 50 μl) was directly added on the top of GHNF dried. More specifically, to investigate the influence of the swelling degree of GHNF on the seeding efficiency, the swelling rate was set to 0, 25, 50, and 100%. The amount of medium specified above was added to the dried GHNF to allow them to swell, and a cell suspension (2×10^6 cells/ml, 50 μl) was added on the top of GHNF. Next, in all experimental groups, the cells were incubated for 3 h, and the number of cells was evaluated as the initial cell attachment [16]. After 3 h incubation, the scaffolds were collected

for assays or submersed in the medium (1 ml/well). Cell culture was performed on an orbital shaker at 90 rpm for 24 and 72 h [17], followed by counting the number of cells proliferation and evaluation of cells distribution. The medium exchange was performed 48 h after seeding. Each experiment at every time point was performed independently for three samples unless otherwise mentioned.

2.5. Evaluation of cell number

The number of cells attached and proliferated in GHNF was determined by the fluorometric quantification of cell DNA according to the assay method reported [4]. Briefly, cell-seeded GHNF were washed with PBS three times and stored at −30 °C until to the assay. After thawing, cells present in GHNF were lysed in a buffer solution (pH 7.4) containing sodium dodecyl sulfate (0.2 mg/ml), and 30 mM saline—sodium citrate (SSC) at 37 °C for 24 h with occasional mixing (300 rpm). The cell lysate (40 μl) was put in a 96 well black flat bottom plate. After that the dye solution (160 μl) (composition: 30 mM SSC and Hoechst 33258 dye [1 μg/ml]), the fluorescence intensity of the mixed solution was measured with a fluorescence spectrometer (SpectraMax; Molecular Devices, California, USA) (excitation, 355 nm; emission, 460 nm). A calibration curve between DNA and cell number was prepared by using cell suspensions with different cell densities.

2.6. Evaluation of cell distribution in GHNF

The GHNF with cells were fixed with 4% paraformaldehyde solution for 30 min at 37 °C. After placing overnight in 30% sucrose/PBS solution, specimens were embedded in an optimal cutting temperature compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan), and frozen by the liquid nitrogen. The frozen samples were sectioned at 10 μm-thick using a cryotome (CM3050S, Leica Microsystems, Wetzlar, Germany). The five sections at the center portion of GHNF were prepared, and pasted on to a slide glass, immersed in 100% ethanol for 10 s, and dried overnight with a circulator. Then, the samples were stained with hematoxylin and eosin followed by sealing with a water soluble encapsulant (Aquatex; Merck Ltd., Darmstadt, Germany). Prepared specimens were examined under a microscope (BZ-X710, Keyence Co. LTD., Tokyo, Japan), followed by the evaluation of cell number and distribution in GHNF by the Image J program (Public Domain Image Processing Program, National Institutes of Health, Bethesda, MD). The portion of GHNF section was divided into an inner and an outer area. The area ratio of the inner to the outer was 1: 2, and the portion of about 500 μm depth from the scaffold surface was defined as the inner area. The cell counting was performed manually for the sections segmented images for each sample. The number of cells was expressed as the number of cells per unit area (cells/mm^2).

2.7. Measurement of lactate dehydrogenase activity

As a measure of cell damage during the seeding process, lactate dehydrogenase (LDH) level was measured by using a Cytotoxicity LDH Assay Kit-WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). After 3 h incubation, GHNF were placed into each well of 48-well multi culture plate, and then 300 μl of medium was added, followed by 30 min incubation. Next, 50 μl of culture supernatant was added into each well of 96-well multi culture plate. The working solution of the kit (50 μl) was added and incubated for 30 min at room temperature under a light shielding condition. Then, 25 μl of stop solution was added, and the absorbance at 490 nm was measured with a fluorescence spectrometer (SpectraMax; Molecular Devices, California, USA). As a high control, 300 μl of the medium and 30 μl of the lysis buffer were added to GHNF

where cells were seeded after 100% pre-swelling. As a low control, the same assay procedure was performed for only the medium.

2.8. Statistical analysis

All the data were statistically analyzed and expressed as the mean $\pm$ the standard error of the mean. The data were analyzed by student t-test to determine the statistically significant difference while the significance was accepted at $p < 0.05$.

3. Results

3.1. Cell number and distribution in GHNF

Fig. 1 shows the number and distribution of cells in GHNF after the pre-swollen and dried seeding. After 3 h, most of cells were localized in the space between fibers. With the incubation time, cells got attached onto the fiber being in the spreading shape (Fig. 1A and B). Irrespective of the seeding methods, the cells were distributed uniformly inside GHNF (Fig. 1C). The distribution pattern was similar between 3 h and 24 h incubations. The cell number ratio of inner site to the total was about 40 and 60%, for pre-swollen and dried seeding methods, respectively. The pre-swollen method showed a higher ratio of inner area. The number of cells significantly increased with time. There was no difference in the cell number ratio of inner to outer area over the time period studies. However, the seeding methods affected the cell number. The pre-swollen method showed a higher seeding efficiency and proliferation of cells than dried seeding method.

3.2. Influence of pre-swelling treatment on the efficiency of cell seeding

Fig. 2 shows the number of cells in GHNF as a function of pre-swelling rate. The seeding efficiency depended on the pre-swelling rate. The highest number of cells remaining in GHNF was observed at the rate of 25%. Fig. 3 shows the LDH activity of cells after seeding into GHNF different pre-swelling rates. As the pre-swelling rate increased, the LDH activity decreased significantly.

4. Discussion

This study demonstrates that the simple static seeding allowed cells to distribute uniformly inside GHNF (Fig. 1). It is reported that different seeding methods of centrifugation, diffusion, permeation, rotation and capillary force seeding have been tried for the uniform cell seeding [18—21]. However, for GHNF, such modified methods are not necessary to achieve a homogeneous cell seeding. Compared with the seeding method previously reported, the present method is a very simple and user-friendly.

In addition, the pre-swelling process of GHNF affected the seeding efficiency of cells. The pre-swelling procedure would give cells a better environment of attachment (Fig. 1C). This result indicates the possibility that the scaffold swelling may be one of the factors contributing to enhance the cell adhesion [22]. It is well known that the cell proliferation depend on the amount of water retained around cells [23]. On the other hand, no researches have been reported so far on the effect of pre-swelling rate on the initial cell seeding efficiency.

In this study, to further evaluate the effect of pre-swelling on the cell seeding, different pre-swollen scaffolds were prepared. The highest seeding efficiency was shown at a swelling rate of 25% (Fig. 2). The hydrogel swelling analysis [24] demonstrates that there are 70% of water in fibers and 30% of water in the space between
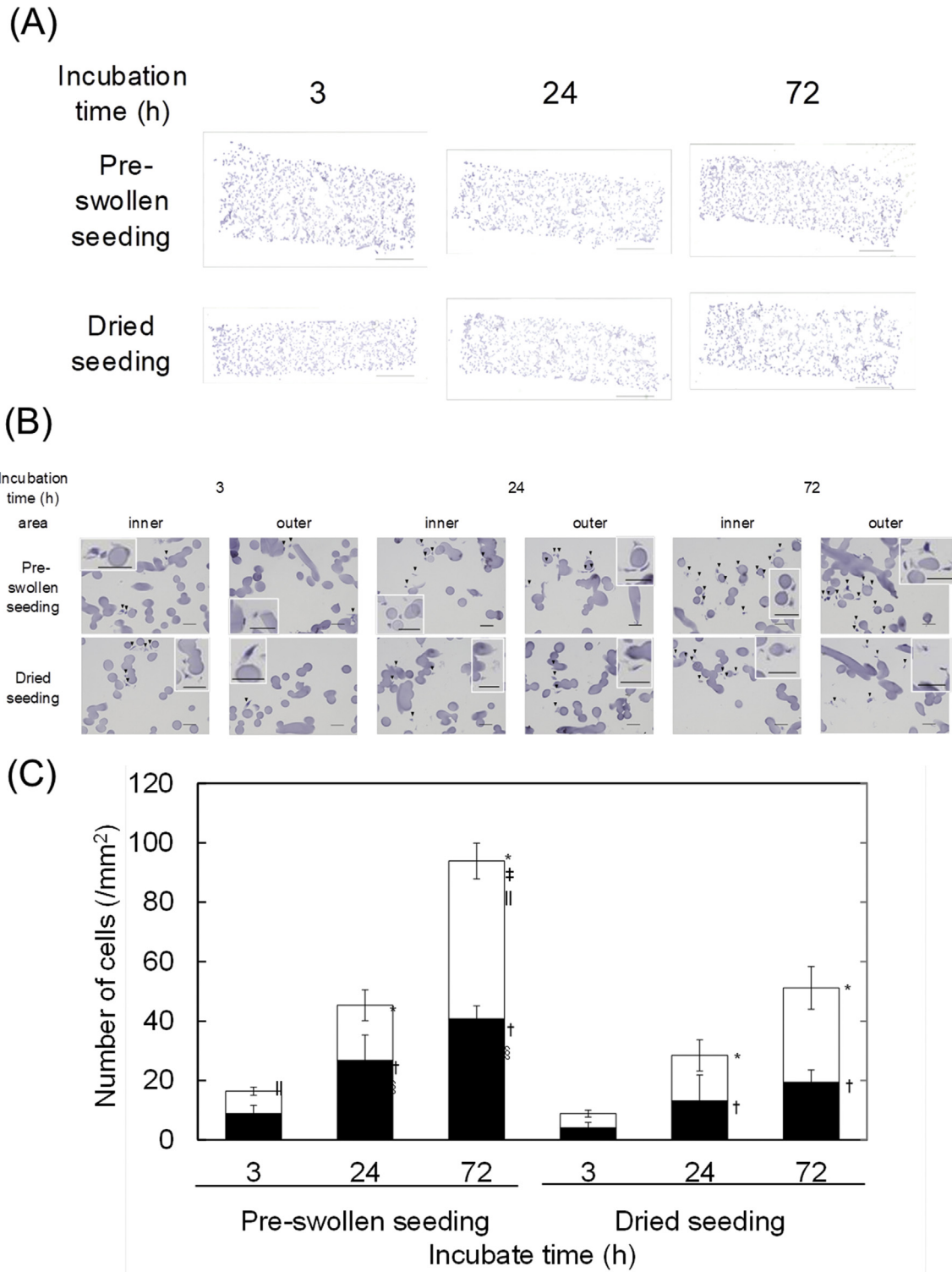
(A)

(B)

(C)

Fig. 1. Number and distribution of cells in GHNF 3 h after seeding or further cultivation for 24 and 72 h by the rotary culture. (A) A whole images of cross sections (Magnification: 20×, scale bar: 1 mm) (B) Detail images of cross sections (Magnification: 20×, scale bar: 50 μm). The black arrows indicate the seeded cells. (C) Time course of cell proliferation and distribution in the outer (□) or inner portion (■) of GHNF after culture for different time periods. *, $p < 0.05$; significant difference against the number of the cells initially attached in the outer portion. †, $p < 0.05$; significant difference against the number of the cells initially attached in the inner portion. ‡, $p < 0.05$; significant difference between numbers in the outer portion at the same time point. §, $p < 0.05$; significant difference between numbers in the inner portion at the same time point. ‖, $p < 0.05$; significant difference between numbers in the total cell numbers at the same time point.

fibers. At the rate of 100 and 50%, since a saturated amount of water is absorbed, it is likely that there is no room to allow the cell suspension to further absorb (Fig. 2), leading to a decreased cell seeding efficiency. On the other hand, at the pre-swelling rate of 0%, the swelling of GHNF and the cell adhesion to the fibers will proceed simultaneously. On the other hand, at the pre-swelling rate of 25%, it is highly conceivable that the fibers were appropriately swollen and there was a certain amount of water in the space
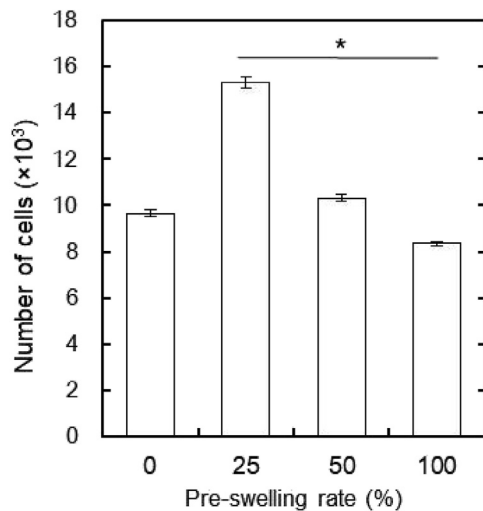
Fig. 2. The number of cells proliferated in GHNF for 3 h as a function of pre-swelling rate. *, $p < 0.05$; significant difference between the two groups.
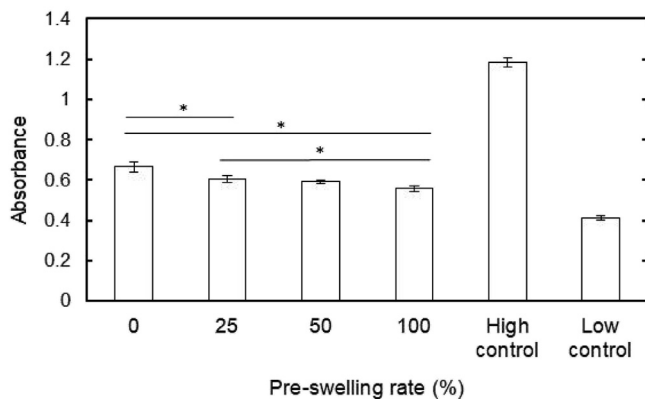

Fig. 3. The LDH activity of cells proliferated in GHNF for 3 h as a function of pre-swelling rate. *, $p < 0.05$; significant difference between the two groups.

between fibers. As the result, the cell suspension added would be trapped and homogeneously distributed into GHNF (Fig. 1). In addition, since the gelatin fibers can absorb water actively, water may be deprived from cells seeded, and consequently the cell membrane damage may occur [25] resulting in an enhanced LDH activity (Fig. 3) [23].

There are two points to be considered for the relationship between the pre-swelling rate and the cell seeding efficiency. The first is the amount of water that can be retained by GHNF, while the second is the relationship of pore size and swelling speed. The cell suspension can be expected to diffuse by leaving the fibers free of moisture and dropping the cell suspension. It is found that the pore size of GHNF is 38 μm in the dry state (data not shown). This pore size is sufficiently larger than the size of a single cell. Therefore, cells seeded would pass through the space between fibers, resulting in a decreased cell seeding efficiency. This may happen before the pore size becomes smaller enough to prevent the cell passing through due to swelling of gelatin fiber of GHNF (Fig. 2) [22].

Taken together, when GHNF still have an ability to absorb water, the cell suspension would uniformly penetrate into the fiber mesh of GHNF, resulting in a homogeneous distribution of cells proliferates. The GHNF are basically composed of cell-friendly gelatin fibers. The space between the fibers is large enough to allow cells

to homogeneously infiltrate into the GHNF just by the simple cell seeding. This may be because the GHNF show a suitable swelling behavior which gives cell a good state of cell initial attachment. In addition, even in the swollen condition, they have a mechanical strength strong enough to maintain the inner porous structure which allows cells to homogeneously infiltrate into them.

References

[1] Li Y, Ma T, Kniss DA, Lasky LC, Yang ST. Effects of filtration seeding on cell density, spatial distribution, and proliferation in nonwoven fibrous matrices. Biotechnol Prog 2001;17:935—44. https://doi.org/10.1021/bp0100878.
[2] Ratheesh G, Venugopal JR, Chinappan A, Ezhilarasu H, Sadiq A, Ramakrishna S. 3D fabrication of polymeric scaffolds for regenerative therapy. ACS Biomater Sci Eng 2017;3:1175—94. https://doi.org/10.1021/acsbiomaterials.6b00370.
[3] Haycock JW. 3D cell culture: a review of current approaches and techniques, vol. 695; 2011.
[4] Hosseinkhani H, Inatsugu Y, Hiraoka Y, Inoue S, Tabata Y. Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly (glycolic acid) fiber. Tissue Eng 2005;11:1476—88.
[5] An J, Teoh JEM, Suntornnond R, Chua CK. Design and 3D printing of scaffolds and tissues. Engineering 2015. https://doi.org/10.15302/j-eng-2015061.
[6] Ma Z, Kotaki M, Inai R, Ramakrishna S. Potential of nanofiber matrix as tissue-engineering scaffolds. Tissue Eng 2005. https://doi.org/10.1089/ten.2005.11.101.
[7] Moutos FT, Freed LE, Guilak F. A biomimetic three-dimensional woven composite scaffold for functional tissue engineering of cartilage. Nat Mater 2007. https://doi.org/10.1038/nmat1822.
[8] Kasoju N, Bhonde RR, Bora U. Preparation and characterization of Antheraea assama silk fibroin based novel non-woven scaffold for tissue engineering applications. J Tissue Eng Regen Med 2009. https://doi.org/10.1002/term.196.
[9] Ahn H, Kim KJ, Park SY, Huh JE, Kim HJ, Yu WR. 3D braid scaffolds for regeneration of articular cartilage. J Mech Behav Biomed Mater 2014. https://doi.org/10.1016/j.jmbbm.2014.01.004.
[10] Liu Z, Tang M, Zhao J, Chai R, Kang J. Looking into the future: toward advanced 3D biomaterials for stem-cell-based regenerative medicine. Adv Mater 2018;30:1—20. https://doi.org/10.1002/adma.201705388.
[11] Drury JL, Mooney DJ. Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials; 2003.
[12] Vunjak-Novakovic G, Obradovic B, Martin I, Bursac PM, Langer R, Freed LE. Dynamic cell seeding of polymer scaffolds for cartilage tissue engineering. Biotechnol Prog 1998;14:193—202. https://doi.org/10.1021/bp970120j.
[13] Nakamura K, Saotome T, Shimada N, Matsuno K, Tabata Y. A gelatin hydrogel nonwoven fabrics facilitates metabolic activity of multi-layered cell sheets. Tissue Eng C Methods 2019;25:344—52. https://doi.org/10.1089/ten.tec.2019.0061.
[14] Medeiros ES, Glenn GM, Klamczynski AP, Orts WJ, Mattoso LHC. Solution blow spinning: a new method to produce micro- and nanofibers from polymer solutions. J Appl Polym Sci 2009;113:2322—30. https://doi.org/10.1002/app.
[15] Ozeki M, Tabata Y. In vivo degradability of hydrogels prepared from different gelatins by various cross-linking methods, 16; 2012. p. 37—41.
[16] Thevenot P, Nair A, Dey J, Yang J, Tang L. Method to analyze three-dimensional cell distribution and infiltration in degradable scaffolds. Tissue Eng C Methods 2008;14:319—31. https://doi.org/10.1089/ten.tec.2008.0221.
[17] Yasuda K, Inoue S, Tabata Y. Influence of culture method on the proliferation and osteogenic differentiation of human adipo-stromal cells in nonwoven fabrics. Tissue Eng 2004;10:1587—96.
[18] Takahashi Y, Tabata Y. Homogeneous seeding of mesenchymal stem cells into nonwoven fabric for tissue. Engineering 2003;9:931—8.
[19] Godbey WT, Stacey Hindy BS, Sherman ME, Atala A. A novel use of centrifugal force for cell seeding into porous scaffolds. Biomaterials 2004;25:2799—805. https://doi.org/10.1016/j.biomaterials.2003.09.056.
[20] Burg KJL, Holder WD, Culberson CR, Beiler RJ, Greene KG, Loebsack AB, et al. Comparative study of seeding methods for three-dimensional polymeric scaffolds. 2000.
[21] Garg RK, Rennert RC, Duscher D, Sorkin M, Kosaraju R, Auerbach LJ, et al. Capillary force seeding of hydrogels for adipose-derived stem cell delivery in wounds. Stem Cells Transl Med 2014;1079—89.
[22] Peter M, Ganesh N, Selvamurugan N, Nair SV, Furuike T, Tamura H, et al. Preparation and characterization of chitosan-gelatin/nanohydroxyapatite composite scaffolds for tissue engineering applications. Carbohydr Polym 2010;80:687—94. https://doi.org/10.1016/j.carbpol.2009.11.050.
[23] Lang F, Ritter M, Gamper N, Huber SM, Fillon S, Tanneur V, et al. Cellular physiology and biochemistry cell volume in the regulation of cell proliferation and apoptotic cell death. Cell Physiol Biochem 2000;10:417—28.
[24] Nichol JW, Koshy ST, Bae H, Hwang CM, Yamanlar S, Khademhosseini A. Cell-laden microengineered gelatin methacrylate hydrogels. Biomaterials 2010;31:5536—44. https://doi.org/10.1016/j.biomaterials.2010.03.064.
[25] Clegg JS, Seitz P, Seitz W, Hazlewood CF. Cellular responses to extreme water loss: the water-replacement hypothesis. Cryobiology 1982;19:306—16. https://doi.org/10.1016/0011-2240(82)90159-6.

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30 September 2019