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Effects of rotational culture on morphology, nitric oxide production and cell cycle of endothelial cells

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ABSTRACT: Devices for the rotational culture of cells and the study of biological reactions have been widely applied in tissue engineering. However, there are few reports exploring the effects of rotational culture on cell morphology, nitric oxide (NO) production, and cell cycle of the endothelial cells from human umbilical vein on the stent surface. This study focuses on these parameters after the cells are seeded on the stents. Results showed that covering of stents by endothelial cells was improved by rotational culture. NO production decreased within 24 h in both rotational and static culture groups. In addition, rotational culture significantly increased NO production by 37.9% at 36 h and 28.9% at 48 h compared with static culture. Flow cytometry showed that the cell cycle was not obviously influenced by rotational culture. Results indicate that rotational culture may be helpful for preparation of cell-seeded vascular grafts and intravascular stents, which are expected to be the most frequently implanted materials in the future.

Introduction

Cells for three-dimensional culture are frequently seeded for tissue engineering on the surface of carriers such as scaffolds and stents (Wu *et al*., 2008; Qiu, 2007). The limitations of static culture, as the traditional cell culture method, have been overcome to some extent by bioreactors (Martin *et al*., 2004). However, a major problem is the difficulty for endothelial cells to adhere onto vascular stents (Shen *et al*., 2009). Although adherence should depend more on surface chemistry than on the culture method, the cells in static culture are distributed asymmetrically and grow poorly on carriers (Srivastava *et al*., 2010; Zhang *et al*., 2006; Giannoglou *et al*., 2008). In recent years, biological reaction devices enabling rotational culture for dynamic cell culture have been applied more because they promote cell adhesion, improve substance-transmission rate, and contribute lower shear stress (Shaw, 2004; Asnaghi *et al*., 2009).

Recently, Siamwala *et al*. (2010) have found that rotational culture could promote both the proliferation and migration of endothelial cells and the angiopoiesis process, and that the mechanisms involved include nitric oxide (NO) as the key compound. Moreover, NO production was increased by activating endothelial inducible NO synthase (iNOS) in endothelial cells in their experiments, and they also confirmed that simulation of microgravity promotes NO-supported angiogenesis through the iNOS-cyclic guanosine monophosphate-protein kinase G pathway. Therefore, the expression of NO can be used to test the efficiency of an endothelial cell culture.

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On the contrary, injured endothelial cells behave differently from normal ones (Shi and Tokunaga, 2004). The functional turbulence results in excessive hyperplasia of the neonatal intima and proliferation of vascular smooth muscle cells (Thyberg *et al*., 1997; Hedin *et al*., 1999;). Also, some reports have found that endothelial cells are related to vascular reconstruction (Siasos *et al*., 2007). The morphology of damaged endothelial cells is anomalous because of change in culture conditions and these cells produce less NO, thus causing resistance to oxidation of low density lipoproteins (LDLs) of the blood vessel wall, elimination of free radicals, and prevention of cell apoptosis (Margariti *et al*., 2006). During the early stages of atherosclerosis, endothelial cells lose control of the cell cycle because of the presence of oxidized-LDL (Zhang *et al*., 2005), resulting in the excessive proliferation of vascular smooth muscle cells in the plaque and being further transferred to the vessel intima (Bancroft *et al*., 2002), which plays an important role in the pathological changes that follow. Therapies of atherosclerosis have focused on cell-seeded stents, and rotational culture was seen as a possibility to resolve the problem of seeding cells on the carrier surface (Lane, 2005; Liu *et al*., 2004), but whether rotational culture influences the cells in some aspects, compared to a static culture, is unknown. The current article explores the effects of rotational culture of endothelial cells on cellular morphology, NO production, and cell cycle.

Materials and Methods

Rotational culture device

A novel rotational culture device (Tang *et al*., 2008) (Chinese patent no. ZL 200820097583.4) was used to create a confluent endothelium on the surface of stents for endovascular use. Stents made of 316L stainless steel 1.6-mm diameter, and 18-mm length), kindly provided by Amsino Medical Co., Ltd (Beijing, China), were used in this experiment. Stents were prepared for seeding by coating with 0.4% glutin and 10 μg/mL poly-L-lysine (PLL) by ultrasonic spraying and ultraviolet light sterilization. The stents to be used as cell carriers, were placed in a specially designed rotational tube, set on a rotator (Chongqing Xianfeng Yuzhou Electronic Co. LTD, Chongqing, China), and incubated with the cell suspension. The device rotated on an axis with a low speed, ensuring slight movements of the tube and allowing adhesion of the cells on the surface of the stents. This rotational culture device could provide a culture environment with both a relatively low shear force and the necessary three-dimensional (3D) interactions among cells; it is therefore suitable for culture of vascular endothelial cells *in vitro*.

Except in those cases designed only to observe morphology, the cells were divided into two groups. One was the rotational culture group; the other was the static culture group. Each treatment was replicated thrice.

Endothelial cells grow on the stents: rotational vs. static culture

Human umbilical vein endothelial cells were obtained from the Medical College of Nanhua University, China, and were grown to confluence in T-75 polystyrene flasks (Becton, Dickinson and Company, NJ, USA) with endothelial basal medium (1640, Hyclone, UT, USA), supplemented with 10% fetal calf serum (Cambrex Corporation, IA, USA) and an antibiotic/antimycotic solution (Gibco, CA, USA). Endothelial cells were cultured in an incubator with 95% air/5% CO_2 at 37ºC and were used at passage 3-6 for all experiments. The endothelial cells were detached from the flasks using trypsin (0.25% trypsin for 2-3 min at 37ºC and neutralized with fetal bovine serum), resuspended in serum-free media, and counted using a counting plate to determine cell density (1.0×10^5 cells/cm^2, 0.5×10^6 cells/cm^2, and 1.0×10^6 cells/cm^2). The stents were fixed in an organic glass tube (approximately 10 cm in length), which was filled with the cell suspension, and placed on the rotational equipment for 6 h of rotational culture at 0.4 rpm, with a cell density of 1×10^6 cells/mL at 37ºC and 5% CO_2, which was considered the best condition for cell adhesion to the surface of stents based on a previous study (Tang *et al*., 2008).The same conditions were applied to the static culture group, but at 0 rpm.

Observation of cell morphology on the stents

After static and rotational culture for 6 h, the stents were taken out and were observed under an optical microscope (IX81; Olympus, Tokyo, Japan). Then the cells on the stents were fixed by glutaraldehyde (2.5% in PBS), dehydrated in ethanol before vacuum freeze-drying and gold coating, and observed under a scanning electron microscope (Quanta 600F, FEI Company, USA). Cell immunofluorescent staining was performed by using primary antibody against rabbit vascular endothelial growth factor (VEGF; Boster, China), and the

cyanine-labeled secondary goat anti-rabbit IgG antibody (Boster, China). Endothelial cells grown on the stents were detected by fluorescence microscopy (IX81; Olympus, Tokyo, Japan).

Rotational and static culture of epithelial cells without stents

Endothelial cells were detached and resuspended in culture medium (1×10^6 cells/mL) in organic glass tubes, and were cultured for 12, 24, 36, and 48 h at either 0.4 rpm or 0 rpm.

Determination of NO Production

After cell culture for 12, 24, 36, and 48 h, a NO Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to determine absorbancy of the cells of the two groups and to calculate production of NO by the endothelial cells.

The following formula was used to calculate the NO production by endothelial cells based on the absorbance detected in the experiment:

$$NO\ \text{Concentration}\ (ng/ml) = \frac{OD_{\text{sample}} - OD_{\text{blank}}}{OD_{\text{standard}} - OD_{\text{blank}}} \times 100\ (nmol/ml) \times 30\ (ng/nmol) \times Dilution$$

Determination of cell cycle stages

Before starting either rotational or static culture, the endothelial cells were synchronized. The cells were cultured in the medium developed by Moore *et al*. at Roswell Park Memorial Institute, termed RPMI 1640, without serum for 24 h in a CO_2 incubator at 37ºC and 5% CO_2, digested, fixed, and observed using routine procedures after rotational and static cultures. The cell suspension was centrifuged at 800 rpm for 10 min, washed with phosphate buffered saline, fixed with 70% ethanol after discarding the lipid-containing supernatant, and the cell cycle stages were determined using a flow cytometer (FACSCalibur Becton Dickinson, Beckman Coulter, Miami, FL, USA).

Statistical Analysis

The data in the text were expressed as mean ± SD. Student's t-test was used to compare the differences between two groups. ANOVA was used for multigroup comparisons, followed by the S-N-K method as post-hoc test.

Results

Cell morphology

The morphology of endothelial cells grown on the surface of stents by both rotational was observed under optical, fluorescence, and scanning electron microscopes. Normally endothelial cells under static culture conditions formed a single layered cobblestone pavement of the stent surface (Fig. 1 A, C and E), but shifted to form a multiple-layered cover characterized by clone-like cell groups after rotational culture (Fig. 1 B, D and F).

Determination of NO production

After the cells were cultured by rotational and static cultures for 12, 24, 36, and 48 h, NO production by the cells in both groups was calculated and compared. Statistical analysis showed that NO production was not significantly different between the rotational and static culture groups when cultivated for 12 and 24 h; however, when the cells were cultivated for 36 and 48 h the

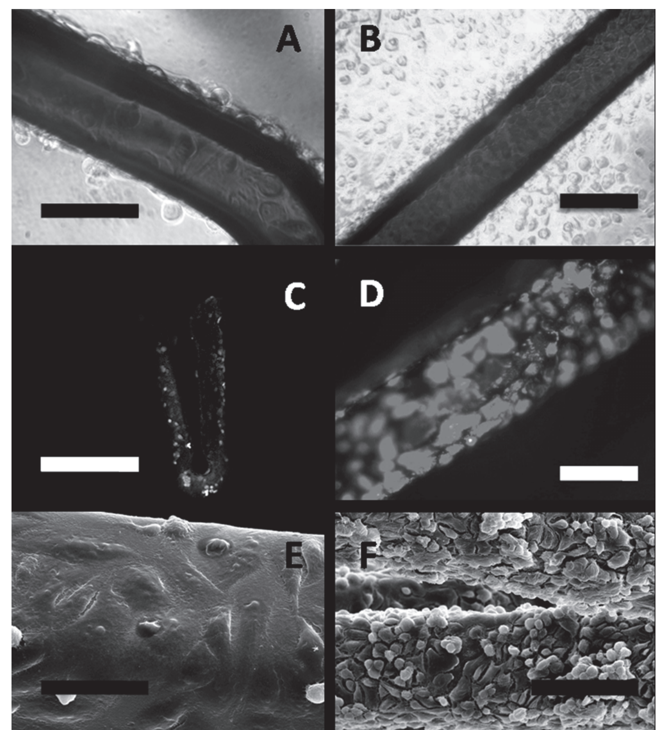

FIGURE 1. Endothelial cells grown in static culture (A bar=100μm, C bar=200 μm, E Bar=50 μm) and rotational culture conditions (B, D, F bars=100 μm) on the surface of stents, detected by optical microscope (**A** and **B**), fluorescence microscope (**C** and **D**), and scanning electron microscope (**E** and **F**).

difference in NO production between static and rotational cultures became statistically significant (P <0.01). NO production by the rotational culture group at 36 and 48 h increased by 37.9% and 28.9%, respectively, as compared with the static culture group.

NO production profiles of the two groups at different time points have also been compared (Fig. 2A and B). The results showed that NO expression of the two groups depended on the culture duration, and the rate continuously decreased for 24 h but increased with time when the duration of the cell culture was more than 24 h; moreover, the upward trend of the rotational culture group was stronger than that of the static culture group.

Determination of cell cycle stages

The endothelial cell cycle stages were determined by a flow cytometry in all groups (Fig. 3). After the cells were rotationally cultured for 12 and 24 h, no significant differences were observed in the cell cycle. However, between 24 and 36 h, the cell cycle changed remarkably, especially at the G1 and S stages, significantly increasing and decreasing, respectively (Fig. 3C and D). In addition, no significant differences were observed between 36 and 48h. Actually, the cell cycle showed a profile similar to that of NO production.

The situation in the static culture group was similar to that of the rotational culture group (Fig. 3). No significant differences were observed between profiles at 12 and 24 h, and 36 and 48h; nevertheless, in the period from 24 to 36 h, the cell cycle changed remarkably. Moreover, compared at the corresponding times, the data showed that the difference between the rotational and the static culture groups was not statistically significant, which showed that rotational culture had little influence on the endothelial cell cycle.

Discussion

The endothelial cells obtained by rotational culture, compared with those obtained by static culture, were probably influenced by a few factors, which may have resulted in the significant differences of morphology

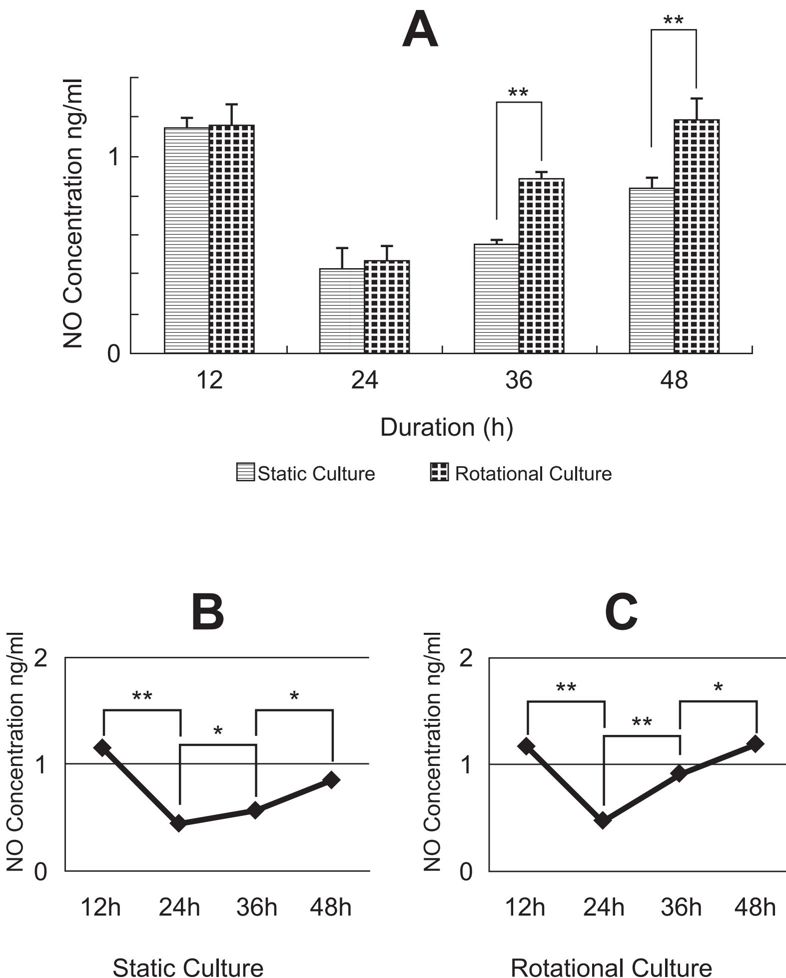

FIGURE 2. NO production in rotational and static culture. (**A**). Bars represent the mean ± SD from three independent experiments. The NO production changed with time in rotational culture group (**B**) and static culture group (**C**). Statistically significant differences: * *P*<0.05, ** *P*<0.01.

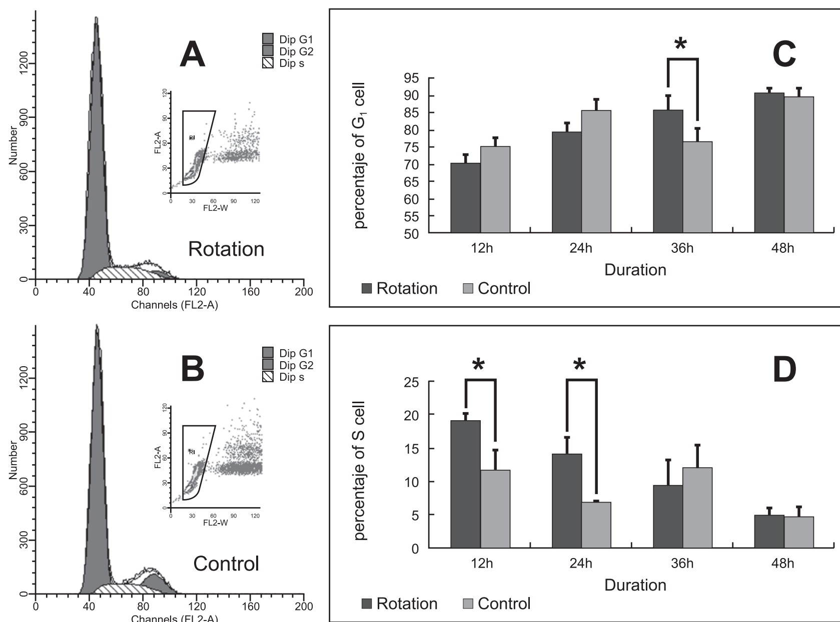

FIGURE 3. Cell cycle in rotational (**A**) and static culture group (**B**) as determined by flow cytometry. The cell cycle changed remarkably, especially at the G1 stage and S stage (**C** and **D**, respectively); however, no signifcant differences were observed between the rotational and the static culture groups. Results are mean ± SD from three independent experiments. Statistically significant differences: * $P<0.05$.

and NO production between the rotational and static culture groups. First, because of the centrifugal force produced by rotation, the cells in the rotational tube over the horizontal axis became weightless, which may have weakened cell sedimentation resulting from gravity (Saini and Wick, 2003; Marjorie *et al*., 2003). Moreover, the cells below the horizontal axis became overweight, which may have strengthened cell sedimentation (Braun *et al*., 2003). The cells were also in a continuous rotational state; consequently, they were continuously transferred from the weightless to the overweight regions, and the transformation was periodic; this caused the cells to be suspended in the force field with the changing direction. The cells were transformed to another direction before responding to gravity in time. Second, the space in which the endothelial cells lay was full, especially on the top (Cristina *et al*., 2005; Kyaw *et al*., 2004). The cell suspension was in a state of laminar flow (Schiffrin, 2002). Therefore, the cell suspension and the rotational tube together had no relative motion and no collisions with other substances, for example, the wall of the rotating tube (Pilane and Labelle, 2005). This condition provided cells with very low shear stress, which made them maintain their configuration and remain active, without being mechanically damaged. Steady laminar flow and low shear stress also make the cells grow in a clone-like way, which made them easier to polarize and maintain a 3D configuration (McCarty, 2004; Dardik *et al*., 2005; Paszkowiak and Dardik, 2003; Napoli, 2003). The biomechanical factors, including the transformation between the weightless and overweight states, steady laminar flow, and very low shear stress, could promote cell growth, compared to the conditions in static culture. This change could result in changes in gene expression correlated with NO signal transduction, such as that mediated by endothelial NOS, studied in previous research (Streb and Miano, 2003; Saxena *et al*., 2002; Conte *et al*., 2002). This could explain why the difference between rotational and static culture groups was significant and why NO production decreased for 24 h and then increased after 24 h, with the increase in the rotational culture group being stronger. On the contrary, the stimulus of changing condition did not result in any significant difference in the cell cycle between the two groups. Nevertheless, the data showed that rotation had little influence on the cell cycle. However, further studies are needed to clarify whether the expression of genes regulating the cell cycle was influenced by the experimental conditions (Losordo *et al*., 2003).

In tissue engineering, it is necessary for 3D culture to seed cells on the surface of carriers, such as a vascular graft or intravascular stent. By resorting to rotational culture, the problem that cells hardly adhere to the material surface was partly resolved. However, the effects that rotation of the culture would produce on the cells were unknown. This study has explored the effects of rotational culture on NO production and cell cycle, and the results showed that the method of rotational culture could remarkably increase NO production after 24 h and has little influence on the cell cycle, thus proving that rotational culture is an effective means to seed cells on

the surface of a carrier. This may be a convenient approach for solving the problem of in-stent restenosis and atherosclerotic therapy.

The current has been focused on the effects of rotational culture on cell morphology, NO production, and cell cycle of endothelial cells. Compared to the static culture, the cells obtained by rotational culture were round, with contracted borders, and many of them grew in as slightly overlapping clone-like groups. Rotational culture could increase NO production over that of static culture by 37.9% at 36 h and 28.9% at 48 h, and the difference was significant. However, rotational culture had little effect on endothelial cell cycle and would not cause any abnormal cell growth.

Rotational culture has presented powerful prospective applications for cells to be successfully seeded on stents in tissue engineering. In addition, our experiment showed that the effects of rotational culture on morphology, NO production, and cell cycle may give useful insights for preparation of cell-seeded vascular grafts and intravascular stents, which are expected to be the most frequently implanted materials in the future.

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