Effects of cryopreservation on excretory function, cellular adhesion molecules and vessel lumen formation in human umbilical vein endothelial cells

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Abstract. Cryopreservation is widely used in regenerative medicine for tissue preservation. In the present study, the effects of cryopreservation on excretory function, cellular adhesion molecules and vessel lumen formation in human umbilical vein endothelial cells (HUVECs) were investigated. After 0, 4, 8, 12 or 24 weeks of cryopreservation in liquid nitrogen, the HUVECs were thawed. The excretory functions markers (endothelin-1, prostaglandin E1, von Willebrand factor and nitric oxide) of HUVECs were measured by ELISA assay. The expression of intercellular adhesion molecule-1 (ICAM-1) in HUVECs was analyzed using flow cytometry. An angiogenesis assay was used to determine the angiogeneic capabilities of the thawed HUVECs. The results demonstrated that cryopreserved/thawed and recultivated HUVECs were unsuitable for tissue-engineered microvascular construction. Specifically, the excretory function of the cells was significantly decreased in the post-cryopreserved HUVECs at 24 weeks. In addition, the level of ICAM-1 in HUVECs was significantly upregulated from the fourth week of cryopreservation. Furthermore, the tube-like structure-forming potential was weakened with increasing cryopreservation duration, and the numbers of lumen and the length of the pipeline were decreased in the thawed HUVECs, in a time-dependent manner. In conclusion, the results of the present study revealed that prolonged cryopreservation may lead to HUVEC dysfunction and did not create stable cell lines for tissue-engineered microvascular construction.

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Introduction

Vascular defects are a common occurrence in bone fractures. The application of orthopedic treatment techniques is limited owing to technical bottlenecks limiting the production/regeneration of high-quality blood vessels (1,2). Following the development of tissue engineering and regenerative medicine, macrovascular damage repair has generally been accomplished, however, microvascular damage repair remains a challenge, which is attributed to the absence of endothelium in artificial blood vessels, and slow blood flow in the microvascular predisposing to thrombus formation (3). Currently, the successful tissue engineering of microvasculature constructs and artificial vessel endothelialization is urgently required for the reconstruction of defects and necrotic lesions of the skeleton.

Human umbilical vein endothelial cells (HUVECs), as a novel cell source for vascular prostheses, have already been applied in cardiovascular and liver tissue engineering associated with tissue-engineered microvascular construction (4,5). Therefore, the preservation of autologous endothelial cells for prospective use in old age can protect against various diseases and graft rejection in tissues defects (6). Emerging evidence shows that cryopreserved/thawed and recultivated endothelial cells are suitable for endothelialization of autologous allograft veins (6). However, the use of cryopreserved HUVECs has also been associated with several problems, including freezing injury, degeneration of morphology and decreased endothelial markers (7). Previous studies have demonstrated that the cryopreservation of complete vessels results in the loss of endothelial cells (8). It is well known that endothelial cells are important in mediating normal vascular physiology, and the partial absence of endothelium leads to the extracellular matrix being in contact with the circulating blood, which generally leads to thrombosis and/or restenosis (9,10). These are the most serious complications observed following angioplasty, stent deployment and prosthetic graft implantation (9).

To further investigate the effects of cryopreservation on HUVECs, the present study compared the excretory function, cellular adhesion molecules and vessel lumen formation of HUVECs following different durations of cryopreservation.

Materials and methods

Cell culture. The HUVECs were prepared from umbilical cord veins by collagenase digestion, as described previously (11). The human umbilical cords specimens (n=6) of newborns were obtained from the Taizhou Hospital of Zhejiang Province, Wenzhou Medical University, (Taizhou, Zhejiang, China) between June, 2015 and September, 2015. The HUVECs were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator (Thermo Fisher Scientific, Inc.) with a 5% CO_2, 95% air atmosphere. The medium was replenished every day. The HUVECs were identified as endothelial in origin by their cobblestone appearance under phase contrast microscopy. This present study adhered to the tenets of the Declaration of Helsinki and ethical approval was provided by the medical ethics committee of Wenzhou Medical University (Taizhou, China). Informed consent was obtained from patients.

Cryopreservation and thawing. The HUVECs cells were harvested by exposure to 0.25% trypsin and 0.02% EDTA (Gibco; Thermo Fisher Scientific, Inc.), and digestion was terminated with complete medium, following which the supernatants were removed by centrifugation (150 x g for 7 min at 4˚C). A total of $1x10^7$ cells were suspended in 3% w/w dimethyl sulfoxide (Me_2SO; Gibco; Thermo Fisher Scientific, Inc.) on ice for 10 min, followed by an additional incubation with 10% Me_2SO (final concentration) for a further 20 min on ice. The cells were then cooled at a rate of 1˚C/min in a freezing container (Nalgene, Hereford, UK) to 80˚C and, after 24 h, the cells were stored in liquid nitrogen (-196˚C). After 0, 4, 8, 12 or 24 weeks of cryopreservation in liquid nitrogen at -196˚C, the HUVECs were thawed in a 37˚C water bath for 1-2 min. Oscillation of the cryopreserved tube was performed until the cell suspension had completely thawed (12).

ELISA assay. The HUVECs were plated and treated in 96-well plates, and were centrifuged to obtain the supernatant. The levels of endothelin-1 (ET-1), prostaglandin E1 (PGE-1), von Willebrand factor (vWF) and nitric oxide (NO) were measured in the supernatant using an ELISA kit (Immutopics, Inc., San Clemente, CA, USA) at 450 nm using an ELISA reader (BioTek Instruments, Inc., Winooski, VT, USA), according to the manufacturer's protocol.

Flow cytometry. The HUVECs ($5x10^5$) were collected in cold PBS and EDTA (5 mM) followed by incubation with anti-ICAM-1 (cat. no. HA58; 1:1,000; BD Biosciences, San Jose, CA, USA) for 1 h at 37˚C. The LSR II system (BD Biosciences) was used for fluorescence acquisition and data were analyzed with FACSDiva software (version 6.1.3; BD Biosciences). The samples were gated using a forward scatter and side scatter gate eliminating debris. The fluorescent parameters were set based on the unstained controls.

Immunofluorescence staining. The HUVECs ($5x10^5$) were stained with anti-CD31 antibody (ab28364; 1:100; Abcam, Cambridge, UK), anti-CD34 antibody (ab8536; 1:100; Abcam) and anti-factor VIII antibody (sc-27,649; 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight, as described previously (13). The $CD34^-/CD31^+$ cells were isolated from the HUVEC suspension using a two-step sorting method with immunomagnetic beads, as described previously (14). Negative control staining was performed in parallel with the omission of primary antibodies. All images were obtained using an inverted fluorescence microscope (Leica DM5500B; Leica Microsystems, Inc., Wetzlar, Germany). The uptake of acetylated low-density lipoprotein (LDL) was visualized by immunofluorescence.

Mean vessel density analysis. According to modified version of a previously described protocol (15), equipment for an angiogenesis assay (BD Pharmingen, Franklin Lakes, NJ, USA) was used to determine the angiogeneic capabilities of the thawed HUVECs. In addition, the angiogenic function of the thawed HUVECs stained with calcein (BD Biosciences) was recorded using a fluorescent microscope (Leica DM5500B, Leica Microsystems, Inc.), as described previously (16,17). Images from the fluorescent immunohistochemical analysis were imported into ImageJ analysis software (version 2.1; NIH, Bethesda, MD, USA; http://www. rsbweb.nih.gov/ij/).

Statistical analysis. The data from all experiments are reported as the mean ± standard deviation for each group. All statistical analyses were performed using PRISM version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Intergroup differences were analyzed using one-way analysis of variance, and followed by Tukey's multiple comparison test as a post hoc test to compare group means if significant. P<0.05 was considered to indicate a statistically significant difference.

Results

Morphological observation and identification of HUVECs. The high purity HUVECs were successfully harvested from fetal umbilical cords, and morphological observation of HUVECs was performed using an inverted microscope following culture for 24 h. As shown in Fig. 1A, the purified HUVECs exhibited a typical cobblestone-like endothelial morphology and had high multiplicative ability. In addition, the HUVECs were defined by the expression of CD31, CD34 and factor VIII. The pre-cryopreserved HUVECs were almost 100% positive for CD31 and factor VIII, and ~30% positive for CD34, as demonstrated by immunohistochemistry (Fig. 1B and C). The pre-cryopreserved HUVECs were also examined for endothelial cell phenotype by the uptake of acetylated LDL. As shown in Fig. 1D, immunofluorescence microscopy showed positive staining for Dil-labeled acetylated LDL in the HUVECs.

Effects of cryopreservation on the excretory function of HUVECs. The vascular endothelium is involved in the production of several important substances, which are involved in endothelial dysfunction. ET-1 is characterized as a potent vasoconstrictor and is regulated in the endothelium. NO is the most important vascular relaxing factor, which is also regulated in the endothelium. Alterations in the endothelial production of NO and ET-1 are known to correlate
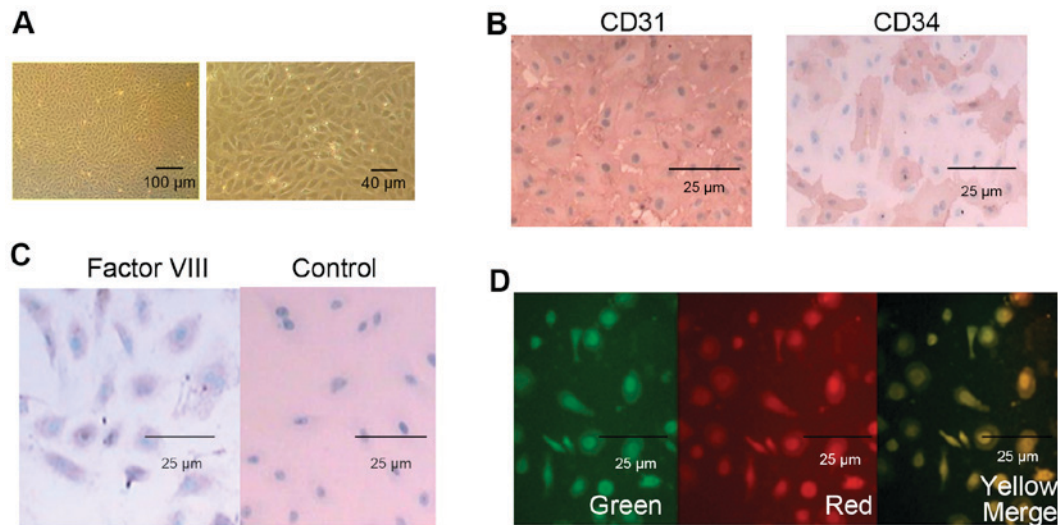
Figure 1. Morphological observation and identification of HUVECs. (A) Bright field microscopy of primary HUVECs at the first passage. HUVECs were stained with (B) anti-CD31 antibody, anti-CD34 antibody and (C) anti-factor VIII antibody immunofluorescence staining. (D) Uptake of acetylated LDL was visualized by immunofluorescence. HUVECs, human umbilical vein endotheleial cells.
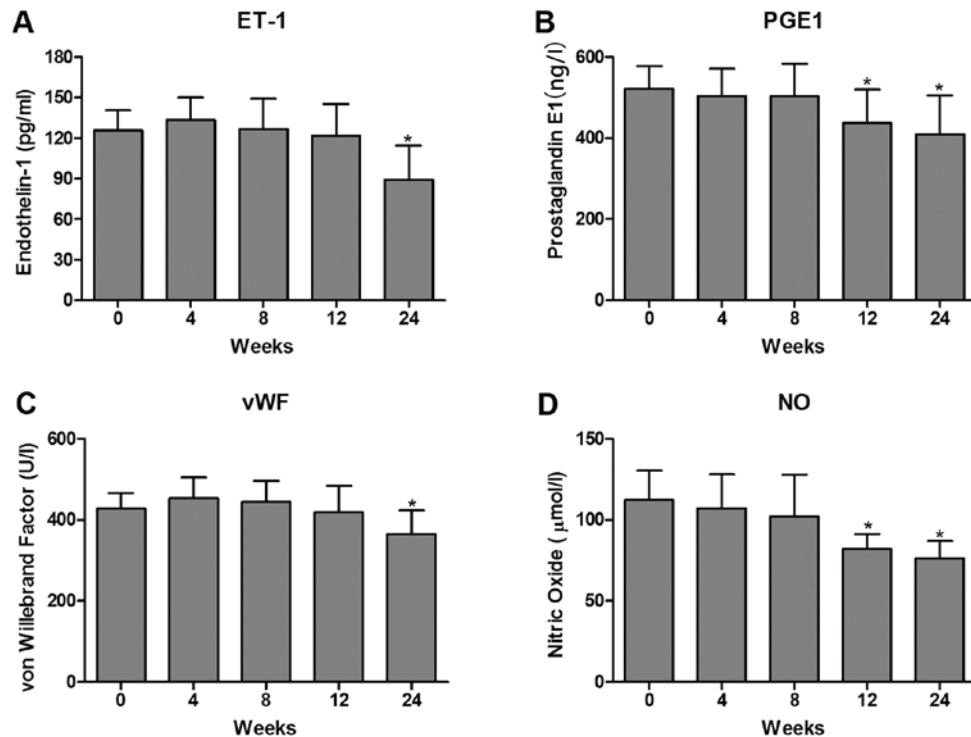

Figure 2. Excretory function of HUVECs. The levels of (A) ET-1, (B) PGE-1, (C) vWF and (D) NO in thawed human umbilical vein endotheleial cells were measured using an ELISA kit. Values are expressed the mean ± standard deviation (n=3 in each group). *P<0.05, vs. control group (week 0). ET-1, endothelin-1; PGE-1, prostaglandin E1; vWF, von willebrand factor; NO, nitric oxide.

with endothelial dysfunction (18). In addition, PGE-1 can effectively protect endothelial cells against oxidative stress induced by hydrogen peroxide, which may depend on the regulation of the expression of NO (19). One such substance, which is synthesized by, and stored in, endothelial cells is vWF. High vWF levels have been shown to be of prognostic value in patients with ischemic heart disease, peripheral vascular disease and inflammatory vascular disease (20). However, there is limited information regarding whether ET-1, PGE-1, vWF and NO are involved in the effects of cryopreservation on the excretory function in HUVECs. In the present study, the levels of ET-1, PGE-1, vWF and NO were measured using an ELISA assay. The results showed that the levels of ET-1, PGE-1, vWF and NO were significantly decreased in the post-cryopreserved HUVECs at 24 weeks. However, no differences were observed in these markers in the post-cryopreserved HUVECs in the first 8 weeks (Fig. 2A-D). These results suggested that prolonged cryopreservation may lead to excretory dysfunction in post-thawed HUVECs.
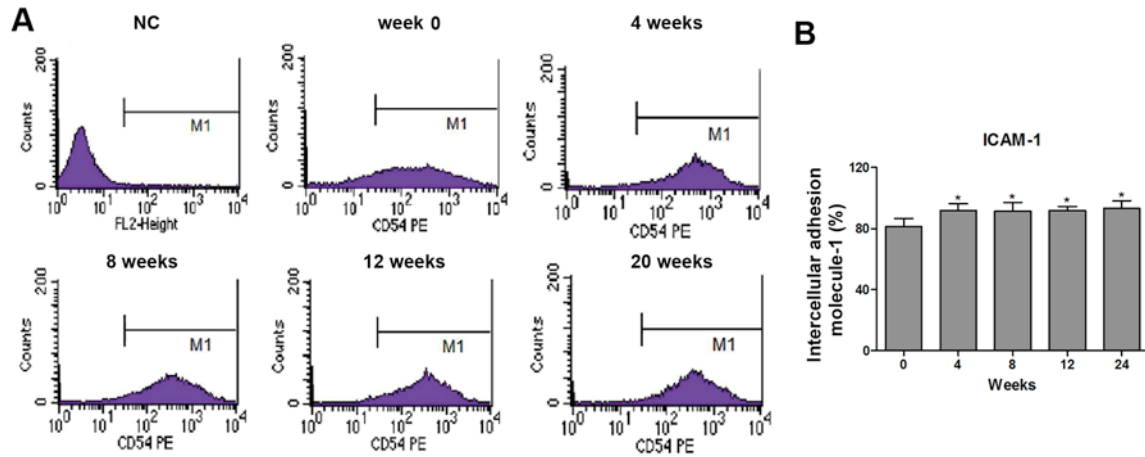
Figure 3. Expression of ICAM-1 in HUVECs. (A) Human umbilical vein endotheleial cells were incubated with anti-ICAM-1 and analyzed using flow cytometry. (B) histograms show the quantitative results. Values are expressed as the mean ± standard deviation (n=3 in each group). *$P<0.05$, vs. control group (week 0). NC, negative control; ICAM-1, intercellular adhesion molecule-1.
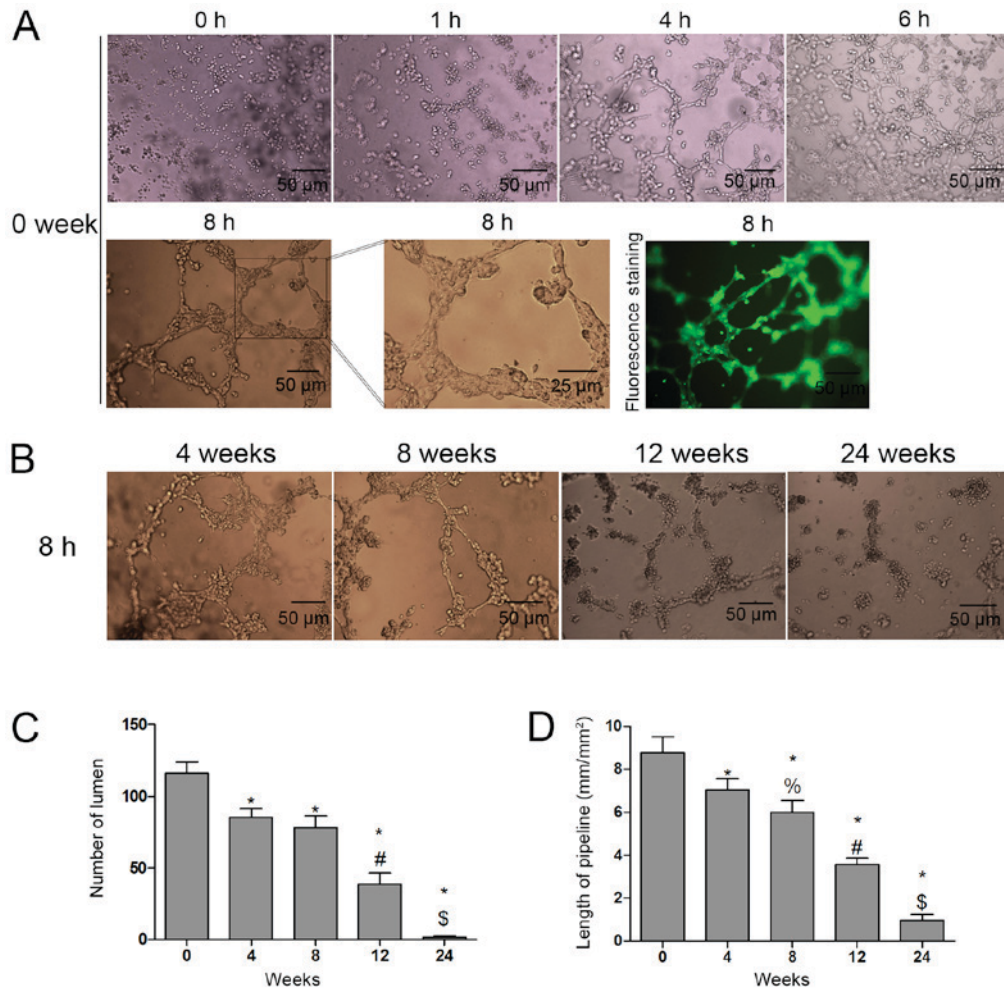

Figure 4. Vessel lumen formation in HUVECs. (A) Angiogenic function of HUVECs (week 0) stained with calcein and visualized using a fluorescent microscope. (B) Angiogenic function of thawed HUVECs (4, 8, 12 and 24 weeks) measured using a fluorescent microscope. (C) Lumen number and (D) pipeline length were quantitatively analyzed using ImageJ analysis software. *$P<0.05$, vs. control group (week 0); %$P<0.05$, vs. thawed HUVECs (week 4); #$P<0.05$, vs. thawed HUVECs (week 8); $$P<0.05$, vs. thawed HUVECs (week 12). HUVECs, human umbilical vein endotheleial cells.

Effects of cryopreservation on the expression of intercellular adhesion molecule-1 (ICAM-1) in HUVECs. A previous study has demonstrated that interactions between ICAM-1 expressed

on endothelial cells and circulating monocytes may be critical for the adhesion of these cells on the vascular endothelium (21). In the present study, the effect of cryopreservation

on the expression of ICAM-1 was also examined. Using flow cytometry, it was demonstrated that the post-cryopreserved HUVECs exhibited marked upregulation in the expression of ICAM-1 (Fig. 3A and B). The cryopreservation-mediated induction of ICAM-1 in the HUVECs was significantly upregulated as early as 4 weeks following treatment, with no differences in the time course of ICAM-1 induction among the cryopreserved groups (Fig. 3B).

Effects of cryopreservation on vessel lumen formation in HUVECs. The numbers of lumen and the length of the pipeline were assessed to indicate the angiogenic function of the frozen and the fresh HUVECs in the second passage. The results showed that the tube-like structures were found in fresh HUVECs with increasing incubation duration and were fully formed at 8 h (Fig. 4A). In addition, the tube-like structure-forming potential was weakened with increasing cryopreservation duration (Fig. 4B). The numbers of lumen (Fig. 4C) and the length of the pipeline (Fig. 4D) were decreased in the thawed HUVECs in a time-dependent manner.

Discussion

Cryopreservation is a valuable technique for preserving cells and tissue materials for regenerative medicine and autologous tissue regeneration, depending on improving technology (6). The present study aimed to investigate the effect of cryopreservation on excretory function, cellular adhesion molecules and vessel lumen formation in HUVECs, which were cryopreserved following a standard protocol. The results demonstrated that cryopreserved/thawed and recultivated HUVECs were unsuitable for tissue engineered microvascular construction or artificial vessel endothelialization. Specifically, the excretory function of HUVECs was significantly decreased in the post-cryopreserved HUVECs at 24 weeks. In addition, the cryopreservation-mediated induction of ICAM-1 in the HUVECs was significantly upregulated from week 4. Furthermore, the potential to form tube-like structures was weakened with increasing cryopreservation duration, and the number of lumen and pipeline length were decreased in the thawed HUVECs in a time-dependent manner.

It has been demonstrated that the cryopreservation of endothelial cells from human umbilical cord vessels offers the opportunity to provide seeding cells for tissue engineering (22). However, certain studies have suggested that the cryopreservation of human saphenous veins is accompanied by endothelial desquamation with loss of anticoagulant function and endothelial healing *in vivo* (8). Current cryopreservation methods universally involve the use of additives and cytoprotectants, which may trigger osmotic damage during the addition and removal of cryoprotectants (17,23,24). As the results of the present study showed, the anabiosis HUVECs lost partial function, which was consistent with previous investigations. However, there is evidence that cryopreserved/thawed and recultivated endothelial cells are suitable for the endothelialization of autologous allograft veins (6). In porcine endothelial progenitor cells, no significant differences in cell viability were found between storage durations of 1, 3, 6, 12 or 18 months following cryopreservation, and no significant differences in cell

proliferation or migration were detected between fresh cells and cells cryopreserved for up to 18 months (17).

The aims of the present study were to elucidate the potential of HUVECs as a tool, in terms of the feasibility of creating stable cell lines, maintaining them over a long time period and reusing them following cryopreservation. The expression of the adhesion marker, ICAM-1, and vessel luminal formation potential in cell culture over time (continuous cultivation time of 6 months) were evaluated following cryopreservation. Prolonged cryopreservation did not create stable cell lines for tissue-engineered microvascular construction and artificial vessel endothelialization. ICAM-1 in the HUVECs was significantly upregulated following cryopreservation. ICAM-1 is known to mediate leukocyte and platelet-endothelial cell interactions in the vasculature under physiological and pathological conditions (25). In addition, ICAM-1 has been shown to be vital in the development of vascular inflammation and endothelial dysfunction, which is associated with the pathogenesis of multiple cardiovascular diseases (26,27). The results suggested that prolonged cryopreservation may lead to HUVEC dysfunction. However, the tube-like structure-forming potential was weakened with increasing cryopreservation duration. The numbers of lumen and pipeline length were decreased in the thawed HUVECs in a time-dependent manner.

In conclusion, the results of the present study revealed that prolonged cryopreservation may lead to HUVEC dysfunction and did not create stable cell lines for tissue-engineered microvascular construction. However, it has previously been documented that minimal cell damage occurs during the storage period when using optimal cryopreservation methods (28). Therefore, the establishment of an optimal condition for cryopreservation is an urgent requirement of future investigations.

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