Preconditions for warm organ preservation
Post, I.C.J.H.

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Endothelial cell preservation at hypothermic to normothermic conditions using clinical and experimental organ preservation solutions

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Abstract

Endothelial barrier function is pivotal for the outcome of organ transplantation. Since hypothermic preservation (gold standard) is associated with cold-induced endothelial damage, endothelial barrier function may benefit from organ preservation at warmer temperatures. We therefore assessed endothelial barrier integrity and viability as function of preservation temperature and perfusion solution.

Human umbilical vein endothelial cells (HUVEC) were preserved at 4 to 37 °C for up to 20 hours using Ringer’s lactate, histidine-tryptophan-ketoglutarate-solution, University of Wisconsin (UW) solution, Polysol, or endothelium culture growth medium (ECGM). Following preservation, the monolayer integrity, metabolic capacity, and ATP content were determined as positive parameters of endothelial cell viability. As negative parameters, apoptosis, necrosis, and cell activation were assayed. A viability index was devised on the basis of these parameters.

HUVEC viability and barrier integrity was compromised at 4 °C regardless of the preservation solution. At temperatures above 20 °C, the cells’ metabolic demands outweighed the preservation solutions’ supporting capacity. Only UW maintained HUVEC viability up to 20 °C. Despite high intracellular ATP content, none of the solutions were capable of sufficiently preserving HUVEC above 20 °C except for ECGM.

Optimal HUVEC preservation is achieved with UW up to 20 °C. Only ECGM maintains HUVEC viability at temperatures above 20 °C.
Introduction

Graft function after transplantation is coupled to post-preservation endothelial function and barrier integrity. The endothelium does not only provide a dynamic barrier in metabolic trafficking but also controls vasomotor activity and regulates innate immune responses, coagulation, and neovascularization. Loss of endothelial integrity during organ preservation therefore triggers microcirculatory hypoperfusion and, more importantly, results in an activated state of the endothelium. The activated state promotes intravascular coagulation and immunological complications that may lead to graft rejection.

Most preservation-induced damage can be attributed to hypothermic (4 °C) organ preservation, with static storage being the current gold standard. Hypothermia decreases the intracellular degradation of metabolites or nutrients by reducing the cell’s metabolic rate by 70 - 90 %, resulting in sufficient energy and nutrient levels during the preservation period. However, the negative influences of hypothermia on membrane-fluidity, the cytoskeleton, intercellular contacts, vasomotor activity, and cell survival are detrimental during prolonged storage.

Therefore, application of (sub)normothermic (20 - 37 °C) preservation (SNP) has been proposed to bypass hypothermia-related effects and consequently improve organ function and transplantation outcome. Preservation of metabolically active cells during SNP, however, requires extensive metabolic support. Even with the advent of novel, nutrient-enriched preservation solutions, sustaining cell metabolism during SNP might prove cumbersome. In addition, current preservation solutions used for SNP must contain components like blood derivates or artificial oxygen carriers to maintain organ and endothelial function. These components are, to date, not amenable to widespread clinical implementation.

In view of the importance of endothelial integrity for post-transplantation outcome and the anticipation of a shift from hypothermic to (sub)normothermic organ preservation, this study aimed to investigate the temperature- and preservation solution-dependent effects on endothelial cell integrity, viability, and activation using human umbilical vein endothelial cell cultures. We used several clinical and experimental preservation solutions, including Ringer’s lactate, histidine-tryptophan-ketoglutarate solution, University of Wisconsin solution, Polysol, and an endothelial culture medium at hypothermic to normothermic (4, 15, 20, 28, or 37 °C) cell culture conditions. The results provide insight in the functionality of endothelial cells during hypothermic preservation and SNP, and identify limitations of the currently employed preservation solutions on
endothelial function under (sub)normothermic conditions.

Materials and Methods

Human umbilical vein endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained at the obstetrics department in our institution. Umbilical veins were cannulated within 36 h after retrieval, flushed with calcium-free medium (142 mM NaCl, 6.7 mM KCl, 3.36 mM HEPES in demineralized water (DW), ingredients from Sigma-Aldrich, St. Louis, MO), and perfused with 5 to 15 mL of 0.04 % Liberase (Roche, Basel, Switzerland) in phosphate buffered saline (PBS, Fresenius Kabi, Graz, Austria). The cords were incubated for 30 min at culture conditions (95 % Air, 5 % CO₂, 37 °C). Next, the veins were opened, flushed with 20 % fetal calf serum-enriched (FCS, Lonza, Basel, Swiss) Williams medium E (WE, Lonza), and the effluent collected and washed twice in 20 % FCS-enriched WE (10 min, 400× g, 4 °C). The cell pellet was resuspended in endothelial cell growth medium (ECGM, Promocel, Heidelberg, Germany) and seeded in culture flasks (T25 Primaria, BD Biosciences, Franklin Lakes, NJ). Upon reaching a confluent first passage, the HUVEC were cryopreserved in liquid nitrogen for at least one week in WE containing 20 % FCS and 20 % DMSO (Sigma-Aldrich). At least two isolated aliquots of cryopreserved HUVEC were thawed prior to each experiment. The HUVEC were washed (10 min, 400× g, 4 °C) in 10 % FCS-enriched WE, cultured in ECGM, and used at a confluent third passage (P3 HUVEC). The experimental design for P3 HUVEC is provided in Figure 1.

Electric cell-substrate impedance sensing

P3 HUVEC were incubated with 1 mL Accutase (Innovative Cell Technologies, San Diego, CA) for 10 min. The collected cells were counted and titrated to approximately 6× 10⁵ cells per well (Casy Counter, Roche), seeded on electric cell-substrate impedance sensing (ECIS) culture arrays (8W10E, Applied Biophysics, Troy, NY) that were pretreated with L-cysteine (10 mM in 0.9 % NaCl, Sigma-Aldrich) and fibronectin (FN, 5× 10⁻³ %, product# F1141, Sigma-Aldrich), and grown in ECGM (300 μL/ well). Next, ECIS arrays were connected to the ECIS console (ECIS Z, Applied Biophysics) and placed in culture conditions for at least 23 h, allowing the cells to form a stable, ECIS-confirmed, confluent monolayer.

After reaching confluence, the medium was aspirated, the wells were washed with 10 mM tris(hydroxymethyl)aminomethane (TRIS) in DW, and 300 μL of one the
Figure 1.
Overview of the experimental setup, preservation solutions and temperatures, and outcome parameters used in the HUVEC assays.

RL, Ringer’s lactate; HTK, histidine-tryptophan-ketoglutarate solution; UW, University of Wisconsin solution; PS, Polysol; ECGM, endothelial cell growth medium.
following solutions was added: Ringer’s lactate (RL, Baxter, Deerfield, IL), histidine-
tryptophan-ketoglutarate solution (HTK, Dr F Köhler-Chemie, Bensheim, Germany),
University of Wisconsin solution (UW, Bristol-Myers Squib, New York, NY), Polysol (PS,
Organoflush, Amsterdam, The Netherlands), or fresh ECGM, all of which the individual
compositions are provided in Table 1. Then, ECIS arrays were transferred to a custom-built
incubator, exposed to 4, 15, 20, 28, or 37 °C for 20 h (n= 3 wells/ solution/ temperature) in a
controlled humidified atmosphere with 95 % air and 5 % CO$_2$ and continuously measured
by ECIS. ECIS data were corrected for fluid specific conductivity at all temperatures to
eliminate conductivity-based influences and expressed as a percentage of monolayer
impedance measured at the end of the 23-h monolayer formation prior to the solution
change (t= 0 h).

Table 1. Composition of solutions used.

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<th>ECGM*</th>
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Endothelial cell preservation at hypothermic to normothermic conditions

Table 1 continued

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**Anti-oxidants (mmol/L)**

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**Osmolarity (mOsm/L)**

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**pH**

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Abbreviations: RL, Ringers lactate; HTK, Histidine-tryptophan-ketoglutarate; UW, University of Wisconsin solution; PS, Polysol; ECGM, endothelium cell growth medium; PEG, Polyethylene glycol; HES, Hydroxyethyl starch

$ (mmol/L): ascorbic acid (0.11), biotin (0.21), Ca-pantothenate (0.004), choline chloride (0.01), inositol (0.07), ergocalciferol (3 \times 10^{-4}), folic acid (0.002), menadione (4 \times 10^{-5}), nicotinic acid (0.004), pyridoxal (0.005), riboflavin (0.003), thiamine (0.03), vitamin A (3 \times 10^{-4}), vitamin B12 (1 \times 10^{-4}), and vitamin E (5 \times 10^{-5})$

$$ (mmol/L): alanine (1.01), arginine (1.18), asparagines (0.08), aspartic acid (0.23), cystine (0.33), glutamic acid (0.34), leucine (0.57), glutamine (0.002), glycine (0.67), isoleucine (0.38), lysine (0.48), methionine (0.30), ornithine (2.00), phenylalanine (0.30), proline (0.78), serine (0.29), threonine (0.34), tryptophan (0.88), tyrosine (0.19), and valine (0.43)$

*personal communication with PromoCell, other ingredients undisclosed
Flow cytometry

Flow cytometry (FACS) was used to determine the extent of apoptosis, necrosis, and endothelial activation after preservation. To this end, P3 HUVEC (n= 4/ solution/ temperature) were seeded on L-cysteine and FN-pretreated culture slides (BD Falcon 8-well CultureSlide, BD Biosciences). After 10- or 20-h preservation at 4, 15, 20, 28, or 37 °C, the medium was aspirated, wells washed with 100 mM TRIS in DW (5 min, 400x g, 4 °C), HUVEC incubated with 75 µL Accutase for 10 min, and washed in 1 % FCS-enriched PBS (PBS+). The pelleted cells were resuspended in PBS+ and divided into two 300-µL aliquots and one 100-µL aliquot for immunoglobulin- and fluorophore-matched isotype control. One 300-µL aliquot was incubated with R-phycoerythrin-conjugated mouse anti-human E-selectin (CD62E-PE) monoclonal antibodies (1:25 dilution, BD Pharmingen, BD Biosciences) and Alexa Fluor 647-conjugated mouse anti-human intercellular adhesion molecule-1 (ICAM-1, CD54-AF647) monoclonal antibodies (1:400 dilution, AbD Serotec, Kidlington, UK) for 20 min at room temperature (RT). The other aliquot was incubated with fluorescein isothiocyanate (FITC)-conjugated annexin V (diluted 1:25 in annexin V binding buffer, BD Pharmingen) and ToPro-3 (1:8000 dilution, Life Technologies, Paisley, UK). Annexin V was incubated with cells for 10 min at RT and ToPro-3 was added just prior to FACS analysis. Ten thousand events were collected for each sample on a Canto 2 flow cytometer (BD Biosciences). FACS data were analyzed using FlowJo software (Tree Star, Ashland, OR), corrected for the background fluorescence intensity derived from the isotype control, and expressed as a percentage of baseline (t= 0 h).

Metabolic activity, intracellular energy, and effector caspases

P3 HUVEC were seeded in FN-coated 24-wells culture plates (Corning Incorporated, Corning, NY). Confluent cells were incubated at 4, 15, 20, 28, or 37 °C for 10 or 20 h and the metabolic activity, intracellular energy content (defined as adenosine triphosphate (ATP) content), and caspase 3 and 7 were assayed in separate cell populations.

Metabolic activity was determined with a tetrazolium salt assay (WST, Roche), which reflects the metabolic capacity of mitochondria. To this end, the medium was aspirated, the wells incubated with WST solution in ECGM at 37 °C, and measured using a Synergy HT microplate reader (BioTek, Winooski, VT) according to the manufacturer’s instructions (λ_{abs}= 440 nm).

ATP content was assessed after 10- or 20-h incubation using a bioluminescence assay kit (CLS II, Roche). After aspiration of the medium, 250 µL of 100 °C TRIS-EDTA-buffer (100 mM TRIS, 4 mM EDTA in DW, pH= 7.75) was added to the wells and kept at 100 °C.
for 2 min. Subsequently, the culture plate was centrifuged (1 min, 400× g, 4 °C), 50 µL of the supernatant aspirated and transferred to white-walled 96-well plates (reference# 3610, Corning), and 50 µL of luciferase reagent was added. Next, the luminescence was measured directly using the fluorescence microplate reader in luminescence mode according to the manufacturer’s instructions.

Caspase 3 and 7 activity levels were determined using a FLICA kit (reference# A20173, AbD Serotec) according to the manufacturer’s instructions (λ_{ex}= 575 nm, λ_{em}= 620 nm). Caspase activation levels were detected in cells after three wash steps with the provided wash buffer.

All data were corrected for the amount of protein per well using a Bradford assay (BioRad, Hercules, CA) and corrected for background absorbance or fluorescence. All parameters are expressed as a percentage of the respective t= 0 h (baseline) value to allow intergroup comparison and calculation of the viability index mentioned in the following section.

**Viability index**

To obtain insight in the cumulative effect of all determined parameters (n= 3/ parameter/ modality) on endothelial function, a viability index was devised according to the following equation:

\[
\frac{(\text{ECIS} \% + \text{WST} \% + \text{ATP} \%)}{((\text{annexin V} \% + \text{FLICA} \%) / 2) \% + \text{ToPro-3} \% + \text{activation} \%)}
\]

The numerator reflects all parameters contributing to maintaining or prolonging endothelial barrier integrity and endothelial function. Therefore, it encompasses the ECIS as gold standard for endothelial barrier integrity, metabolic activity (WST), and ATP content. The denominator includes parameters associated with a compromised endothelial function, either directly through apoptosis (annexin V and FLICA values) or necrosis (ToPro-3-positive cells), or delayed through activation-dependent processes (ICAM-1 and/or E-selectin expression) that are known to impair the transplanted graft’s function. The duplicate outcomes for apoptosis (annexin V and FLICA) were averaged to prevent overrepresentation.

**Light microscopy**

P3 HUVEC were cultured as described above and, after 20-h incubation, imaged
using a phase contrast microscope (Leica DMLB, Leica Microsystems, Wetzlar, Germany) equipped with a Leica DC200 CCD camera that was controlled with QWin software (Leica Microsystems). Furthermore, real time imaging recordings were conducted at 20 °C for 10 h under cell culture conditions to study endothelial barrier dynamics. To this end, primary HUVEC (Lonza) were cultured in culture flasks (TPP, Trasadingen, Switzerland) containing endothelium growth medium-2 (EGM2, Lonza). HUVEC were cultured on FN-pretreated, 24-well glass-bottom imaging plates (Zell-Kontact, Nörten-Hardenberg, Germany). When the monolayer was confluent, cells were washed twice with PBS containing 1 mM CaCl$_2$ and 0.5 mM MgCl$_2$, and incubated with 300-μL RL, HTK, PS, UW, or ECGM. Real time imaging was performed using a Carl Zeiss Observer Z1 microscope (Oberkochen, Germany) with a 20× objective (DIC) with definite focus enabled. Each culture well was automatically imaged every five min during the 10-h incubation.

**Results**

*Endothelial cell barrier integrity*

Endothelial cell barrier integrity was determined using ECIS. The measured impedances represent the tightness of cell-cell contacts, whereby higher electric impedances reflect less permeability and thus more intact endothelial monolayers. All cultured HUVEC exhibited typical monolayer impedances at t= 0 h as exemplified by Figure 2.

![Figure 2](image)
Following 10- or 20-h preservation, a severely compromised integrity was observed for all solutions at 4 °C (Figure 3). In case of RL, barrier integrity never reached baseline values and decreased with increasing temperatures and preservation times. HTK-preserved HUVEC barrier integrity remained around 20 % of baseline impedance values, regardless of incubation temperature and duration. Preservation after 10 h- or 20 h-incubation at 15 °C using PS resulted in an initial impedance increase to respectively 120 % or 90 % of baseline but rapidly dropped with increasing temperatures. UW outperformed all solutions at 15 °C and 20 °C, with impedance levels reaching respectively 180 % and 160 % of baseline values. However, with temperatures above 20 °C, only ECGM was able to maintain endothelial barrier integrity up to 20-h preservation.

Figure 3.
Electric cell-substrate impedance sensing results after 10 (A) or 20 (B) h of human umbilical vein endothelial cells preservation using Ringer’s lactate (RL), histidine-tryptophan-ketoglutarate solution (HTK), University of Wisconsin solution (UW), Polysol (PS), or endothelial cell growth medium (ECGM). The mean ± range impedance of the last hour of preservation is presented as a percentage of baseline.
Figure 4.
The electric cell-substrate impedance sensing results, expressed as a percentual difference from baseline monolayer impedance during 20-hours preservation. Results are expressed as mean (±range) for preservation at A) 4, B) 15, C) 20, D) 28, and E) 37 °C. See Appendix 1, page 208 for the color image.

**Endothelial cell activation and death**

Endothelial cell activation was assessed by means of E-selectin and ICAM-1 expression. An activated state of endothelial cells has been associated with impaired long-
Endothelial cell preservation at hypothermic to normothermic conditions

Annexin V staining, which binds to the cell membrane-expressed phosphatidylserine during apoptosis, was employed as early marker of apoptotic cell death. Additionally, caspase 3 and 7 levels were determined using a FLICA detection kit and necrosis was assessed using ToPro-3, which intercalates into DNA of permeabilized (i.e., necrotic) cells.

Endothelial activation was maximal in HTK-preserved HUVEC after 10-h incubation (170 %, 163 %, and 153 % at 4, 20, and 37 °C, respectively) and 20-h incubation at 4 °C and 20 °C (187 % and 180 %, respectively, Figure 5) compared to baseline. ECGM also resulted in a considerable activation (183 %) after 20-h preservation at 4 °C. Activation of UW-incubated HUVEC peaked to 158 % after 20-h preservation at 20 °C, but the activation state was comparable to baseline at all other conditions. In contrast to all other groups and preservation conditions, the activation observed in the HTK and UW group at 20 °C emanated principally from E-selectin expression (Figure 6, 1C) and to a lesser extent from ICAM-1 expression.

Figure 5.
Endothelial activation was determined by flow cytometry after 10 h (A) or 20 h (B) in Ringer’s lactate (RL), histidine-tryptophan-ketoglutarate solution (HTK), University of Wisconsin solution (UW), Polysol (PS), or endothelial cell growth medium (ECGM). Activation was determined by the extent of E-selectin and intercellular adhesion molecule-1 expression and expressed as the mean (± range) positivity as a percentage of baseline.
Figure 6.

E-selectin (E+), E-selectin and intercellular adhesion molecule-1 (ICAM-1, E+I+), and ICAM-1 (I+) expression were determined by flow cytometry and are presented in the columns from left to right, respectively. Each row represents a different preservation temperature, being: A) 4, B) 15, C) 20, D) 28, and E) 37 °C. Results are expressed as mean (± range) positivity from their respective baseline values. See Appendix 1, page 209 for the color image.
Annexin V-positive but ToPro-3-negative events were predominantly detected at temperatures above 4 °C (Figure 7). However, annexin V-positive events at 4 °C were increased after 20-h preservation with the nutrient-rich UW, PS, and ECGM (134 %, 129 %, and 152 %, respectively). Preservation in RL resulted in increased annexin V binding, with a maximum of 526 % compared to baseline at 28 °C. HTK-induced annexin V binding after 20 h increased from 50 % at 4 °C to 130 % at 15 to 28 °C, with a peak of 242 % at 37 °C. PS showed an increase to 280 % at 20 °C after 20 h, while UW- and ECGM-induced annexin V binding decreased to approximately 90 % of baseline. At 37 °C after 20 h, however, an increase in annexin V binding was detected in UW- (379 %) and PS-preserved HUVEC (295 %) compared to an increase of 157 % in the ECGM group.

Figure 7.
Annexin V as early marker of apoptosis was determined by flow cytometry after 10 h (A) or 20 h (B) with Ringer’s lactate (RL), histidine-tryptophan-ketoglutarate solution (HTK), University of Wisconsin solution (UW), Polysol (PS), or endothelial cell growth medium (ECGM). The mean (±range) positivity (ToPro-3-negative) is presented as a percentage of baseline.
Caspase 3 and 7 were not activated at preservation temperatures up to 20 °C, regardless of the preservation solution (Figure 8). However, a mean increase of 220 % in effector caspases was observed for all solutions at 28 °C, with the maximum activation in the RL group (435 %). Preservation with UW triggered extensive effector caspase activity at 37 °C (520 %), while caspase activity following preservation in RL, HTK, PS, and ECGM was approximately 175 %.

Figure 8.
Effector caspase 3 and 7 levels were determined after 10 h (A) or 20 h (B) in Ringer’s lactate (RL), histidine-tryptophan-ketoglutarate solution (HTK), University of Wisconsin solution (UW), Polysol (PS), or endothelial cell growth medium (ECGM). The mean (±range) levels are presented as a percentage of baseline.

Necrosis was most prevalent in the RL group at 15 °C and 37 °C after 20-h preservation (413 % and 515 %, respectively), but remained around 230 % for the remaining temperatures (Figure 9). HTK- and PS-preserved HUVEC exhibited similar levels of necrotic cell death, between 250 to 350 % of baseline, from 15 °C to 37 °C after 20-h preservation. Preservation with UW at 28 °C and 37 °C was associated with extensive necrosis (357 % and
271 %, respectively), while preservation with ECGM led to a decrease in ToPro-3 positivity from 100 % at 4 °C and 15 °C to 54 % at 37 °C after 20h. The levels detected in the 10-h preservation groups were comparable to those observed after 20-h preservation.

Figure 9.
ToPro-3 as marker of necrosis was determined by flow cytometry after 10 h (A) or 20 h (B) in Ringer's lactate (RL), histidine-tryptophan-ketoglutarate solution (HTK), University of Wisconsin solution (UW), Polysol (PS), or endothelial cell growth medium (ECGM). The mean (± range) positivity is presented as a percentage of baseline.

Metabolic activity and intracellular ATP

Metabolic activity and ATP content were assessed to obtain insight in the metabolic capacity and corollary energy levels of preserved HUVEC.

WST levels were highest after 20 h at 28 °C in the ECGM group (477 %) followed by PS and HTK at 20 °C (457 % and 416 %, respectively, Figure 10). In general, metabolic activity was increased relative to baseline in the RL, HTK, UW, and ECGM groups up to 20 °C following 10- and 20-h preservation. RL-preserved HUVEC displayed a typical decrease in WST levels from 318 % at 4 °C after 10 h to a mean of 150 % after 20 h at 28 °C and 37 °C.
Interestingly, HTK, PS, and ECGM showed a small increase in WST levels at 28 °C compared to the WST levels at 20 °C after 10- and 20-h preservation. Only UW and ECGM were able to yield WST levels of 300 % in cells preserved at 37 °C for 20 h.

Intracellular ATP content was depleted following preservation in RL, regardless of the temperature (Figure 11). Preservation in HTK and ECGM resulted in a small peak of 220 % in ATP content at 15 °C, but remained around baseline at all other temperatures. For UW- and PS-preserved cells, ATP content was equal to baseline levels at 4 °C but increased to 571 % and 379 %, respectively, at 15 °C preservation and to 275 % of baseline levels at 28 °C and 37 °C.

Figure 10.
Metabolic activity was determined with the tetrazolium salt assay (WST) after 10 h (A) or 20 h (B) in Ringer’s lactate (RL), histidine-tryptophan-ketoglutarate solution solution (HTK), University of Wisconsin solution (UW), Polysol (PS), or endothelial cell growth medium (ECGM). The mean (± range) is presented as a percentage of baseline.
Endothelial cell preservation at hypothermic to normothermic conditions

Figure 11.
ATP content (intracellular energy status) was determined by bioluminescence after 10 h (A) or 20 h (B) in Ringer’s lactate (RL), histidine-tryptophan-ketoglutarate solution (HTK), University of Wisconsin solution (UW), Polysol (PS), or endothelial cell growth medium (ECGM). The mean (± range) ATP content, normalized to protein content, is presented as a percentage of baseline.

Viability index

The viability index was devised to obtain insight in factors promoting or compromising endothelial barrier integrity and endothelial function. The index results are shown in Figure 12.

Only preservation with ECGM deterred extensive manifestation of compromising effects and resulted in a temperature-independent net positive effect on barrier function. After 10-h preservation with UW, a similar outcome was obtained. However, the index dropped below 1 after 20-h preservation at 4 °C and 37 °C. UW was best in preserving HUVEC viability at 15 °C and 20 °C. Preservation with PS for 20 h yielded similar results as UW at 15 °C to 28 °C. However, it resulted in a lower viability after 10-h preservation at 4 °C and 15 °C. Preservation in HTK for 10 h was viability promoting at 15 °C and 20 °C, but after 20 h at 15 °C the promoting effects no longer prevailed. RL preservation was detrimental to HUVEC at any temperature after both 10- or 20-h preservation.
Chapter 7

Figure 12.
The viability index of human umbilical vein endothelial cells after preservation in Ringer’s lactate (RL), histidine-tryptophan-ketoglutarate solution (HTK), University of Wisconsin solution (UW), Polysol (PS), or endothelial cell growth medium (ECGM). The index (mean score ± range) is presented for all preservation solutions at all temperatures. When factors promoting endothelial barrier integrity and function outweigh those that compromise endothelial barrier integrity and function, the index value is >1.

Light microscopy

No solution-dependent differences in morphology between HUVEC were observed at 4 °C. All monolayers displayed some gap formation after 20-h preservation at 15 °C, although cell-cell contacts showed increased integrity following preservation with UW, PS, or ECGM (Figure 13). PS-preserved HUVEC showed more pronounced gap formation compared to UW- or ECGM-preserved HUVEC at temperatures above 15 °C. However, PS did not exert as much damage on the monolayer as observed after preservation with RL or HTK. Only ECGM maintained endothelial monolayer integrity at temperatures above 20 °C.

Real time imaging showed marked differences between solutions with respect to HUVEC barrier function and morphology over 10 h at 20 °C. RL preservation resulted in a rapid retraction of the cell plasma membrane with major gap formation and a minor
degree of cell death. During HTK preservation, cell death occurred extensively in the HTK group once the preservation period exceeded 5 h. Preservation with PS resulted in limited gap formation, however, HUVEC were not as active in changing the shape of the cell membrane as observed in cells preserved in UW or ECGM. The HUVEC preserved with UW and ECGM remained viable and exhibited changing cell membrane shape over time, resulting in a net unchanged number of gap formations and a morphologically normal endothelial monolayer.

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Figure 13.
The endothelial monolayer was assessed by light microscopy (10× magnification) after 20-h preservation at each temperature. Preservation of human umbilical vein endothelial cells was performed with Ringer lactate (RL), histidine-tryptophan-ketoglutarate solution (HTK), University of Wisconsin solution (UW), Polysol (PS), or endothelial cell growth medium (ECGM). A clear benefit of preservation with a nutrient-rich solution (UW, PS, or ECGM) can be observed. See Appendix 1, page 210 for the color image.
**Discussion**

Endothelial barrier function, defined as endothelial function and barrier integrity, was assessed by means of an *in vitro* cell preservation model using RL, HTK, UW, PS, and ECGM at 4, 15, 20, 28, and 37 °C. Optimal barrier preservation for 20 h was accomplished using UW at 15 °C and 20 °C. Only ECGM was able to preserve barrier function for 20 h at temperatures above 20 °C.

Barrier function is essential for post-preservation organ function and graft survival. While HUVEC are considered a poor model with respect to allograft-related research due to their higher turnover rate *in vitro*, the metabolism-dependent processes can be accurately assessed. HUVEC have been used in well-established organ preservation-related models of endothelial injury. Moreover, this *in vitro* cell preservation model enables the study of endothelial cell activation, viability, and monolayer integrity under different preservation conditions.

At present, prolonged organ preservation is based on the application of hypothermia that induces preservation-related injury, particularly in marginal donor organs. Causative mechanisms underlying hypothermia-induced endothelial injury are multifactorial and not yet fully elucidated. An important factor appears to be stress on the cytoskeleton, either by flow or hypothermia-induced membrane rigidity, that leads to a cascade of events surpassing the cytoskeleton itself. Disruption of intercellular contacts after hypothermic injury eventually leads to endothelial detachment by undermining the intracellular matrix, of which the implications are shown by our ECIS results.

Our data corroborate that, with rising temperatures, energy-dependent processes reach a turnover point between 4 °C and 15 °C. This turnover coincides with the plasma membrane phase transition of endothelial cells. The implications of this temperature change have been shown previously in bovine aortic endothelial cells. In our study, the turnover point was associated with a sharp increase in E-selectin expression in the absence of ICAM-1 expression, persisting up to 20 °C in HTK- and UW-preserved HUVEC. These high expression levels could be attributed to a previously described cellular stress response initiated at temperatures around 15 °C.

With the temperature dependency of metabolic processes reflected in our results, the ATP data are of particular interest. As intracellular ATP levels are commonly applied as an indicator of cell viability and metabolism, the ATP levels were increased in the 15 to 37 °C temperature range in the energy precursors-containing solutions HTK, UW, and PS. In HTK, the α-ketoglutarate has been shown to be less effective in maintaining ATP
levels than the adenosine present in UW and PS, possibly explaining the difference in ATP between the solutions. However, while high ATP levels are associated with endothelial cell viability up to 24 h under hypothermic conditions, the persistently high ATP content in HUVEC preserved with UW or PS at temperatures above 20 °C did not positively correlate with cell survival in this study. The mismatch in ATP content and viability might be explained by the cell membrane-stabilizing effects of the colloids in UW and PS, causing a decreased cell motility and thus ATP consumption by the cell. Interestingly, ECGM did not result in high intracellular ATP levels, despite the evidently better viability at 37 °C compared to the other preservation solutions. However, a previous study demonstrated that low ATP levels are characteristic of viable cells due to the extensive consumption of ATP. Therefore, as the traditional viability indicators could not accurately determine cell survival, we devised a viability index to gain more in-depth insight into a broader spectrum of (patho)physiological processes. Such an approach has been shown to be helpful previously. The viability index encompassed all the measured outcome parameters and enabled a more accurate assessment of endothelial cell survival.

The presence of high intracellular levels of ATP should favor the induction of apoptosis instead of necrosis. However, annexin V and caspase 3 and 7 levels were only increased after 20 °C preservation with UW or PS in the presence of sufficient levels of intracellular ATP, but not in other groups/conditions where ATP levels were high. During hypothermia, the "flip flop" of phospholipids is disabled by the rigidity of the cell membrane, accounting for the low annexin V binding. In this respect, hypothermic protection against apoptosis has long been established and might explain the predominant occurrence of necrosis instead of apoptosis up to 20 °C. At this temperature, enzymatic cleavage of caspases has been shown to be suboptimal. As a result, preservation of the endothelium has the highest viability score at room temperature conditions (20 °C), combining the advantages of hypothermic preservation with those of (sub)normothermic conditions.

With current organ preservation techniques moving from static cold storage towards hypothermic or (sub)normothermic machine perfusion, intelligent matching of solutions and temperatures can reduce preservation-induced injury. This study was designed to assess perfusate- and temperature-dependent effects on the endothelium in order to optimize (sub)normothermic organ preservation. That preservation at 20 °C is most beneficial for cells in vitro has been demonstrated previously. In our opinion, preservation at 20 °C, i.e., at room temperature, has the advantage of reduced metabolism without an increased inflammatory response and should therefore be investigated further.

In conclusion, preservation of endothelial cell viability in vitro at hypothermic
conditions (at 4 °C) resulted in impairment of endothelial barrier integrity. Maintaining HUVEC viability using preservation solutions at a warmer temperature is possible with UW up to 20 h at 20 °C.
References


