



RESEARCH PAPER

Serum- and albumin-free cryopreservation of endothelial monolayers with a new solution

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ABSTRACT. Cryopreservation is the only long-term storage option for the storage of vessels and vascular constructs. However, endothelial barrier function is almost completely lost after cryopreservation in most established cryopreservation solutions. We here aimed to improve endothelial function after cryopreservation using the 2D-model of porcine aortic endothelial cell monolayers.

The monolayers were cryopreserved in cell culture medium or cold storage solutions based on the 4°C vascular preservation solution TiProtec[®], all supplemented with 10% DMSO, using different temperature gradients. After short-term storage at –80°C, monolayers were rapidly thawed and re-cultured in cell culture medium.

Thawing after cryopreservation in cell culture medium caused both immediate and delayed cell death, resulting in 11 ± 5% living cells after 24 h of re-culture. After cryopreservation in TiProtec and chloride-poor modifications thereof, the proportion of adherent viable cells was markedly increased compared to cryopreservation in cell culture medium (TiProtec: 38 ± 11%, modified TiProtec solutions ≥ 50%). Using these solutions, cells cryopreserved in a sub-confluent state were able to proliferate during re-culture. Mitochondrial fragmentation was observed in all solutions, but was partially reversible after cryopreservation in TiProtec and almost completely reversible in modified solutions within 3 h of re-culture. The superior protection of TiProtec and its modifications was apparent at all temperature gradients; however, best results were achieved with a cooling rate of –1°C/min.

In conclusion, the use of TiProtec or modifications thereof as base solution for cryopreservation greatly improved cryopreservation results for endothelial monolayers in terms of survival and of monolayer and mitochondrial integrity.

KEYWORDS. cryopreservation, cryopreservation solution, endothelial monolayer, mitochondrial fragmentation, TiProtec, vascular storage

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INTRODUCTION

To ensure the availability of vascular grafts for vascular reconstruction/replacement surgery, as well as to allow the storage of products of tissue engineering containing vascular structures,¹ of bio-hybrid prostheses and of organs-on-chips,² adequate storage options have to be provided. For short or intermediate storage, vessels are usually kept at 4°C in buffered salt solutions or in cell culture media. For long-term storage, the only option is cryopreservation. The current gold standard used in vessel banking is cryopreservation in various serum-containing cell culture media (M 199,³ RPMI^{4,5}) with addition of cryoprotective agents (mostly DMSO) and sometimes other additives like human albumin.⁵ However, very modest results are achieved with most current freezing protocols in terms of muscular and especially endothelial function and integrity.^{6–9} In the clinical setting, an impaired endothelial lining induces platelet adhesion and clot formation, so it is highly desirable to preserve the endothelial layer of cryopreserved vessels for transplantation purposes. For vascular constructs in tissue engineering, very little experience exists in the field of storage/cryopreservation.

The vascular storage solution TiProtec[®], which has been developed for cold (4°C) storage of vessels and is based on mechanistic studies, provided marked improvement for cold storage of porcine aortic segments,¹⁰ rat mesenteric arteries and aortae,^{11,12} and human arteries.¹³ TiProtec contains iron chelators to inhibit cold-induced iron-dependent cell injury,^{14,15} glycine and alanine to prevent hypoxic injury,^{16,17} and high chloride and potassium concentrations, which both proved favorable for cold storage of vessels.^{10,11}

Recent results showed that cryopreservation in TiProtec with 10% DMSO – as compared to supplemented cell culture medium with 10% DMSO – improved viability and function of rat hepatocytes after thawing; even better cryopreservation results for hepatocytes, however, were achieved in a chloride-poor modification of TiProtec with balanced sodium/potassium concentrations.¹⁸ TiProtec solution (and modifications thereof) have the additional advantage that they are serum-free and contain no albumin. In contrast to porcine aortic endothelial cells,¹⁰ rat hepatocytes display a chloride-

dependent cold-induced cell injury,^{19,20} i.e. the chloride-poor TiProtec modification is superior to TiProtec for both, cold storage¹⁹ and cryopreservation¹⁸ of rat hepatocytes. Since porcine aortic endothelial cells are better protected in chloride-rich TiProtec at 4°C cold storage,¹⁰ the question arises whether, for these cells, better cryopreservation results can be achieved in the original TiProtec or in chloride-poor modifications.

In this study, we therefore used monolayers of aortic endothelial cells as a simplified 2D-tissue-model, and tested whether TiProtec or the chloride-poor TiProtec modification, which showed best results for rat hepatocyte cryopreservation, are also suitable as base solution for endothelial cryopreservation. In a second step, we transferred the results to porcine aortic segments to assess the effect of cryopreservation in the new solution on (the endothelial lining of) complete vessels.

RESULTS

Cell viability after cryopreservation

In the initial monolayer cultures, virtually no dead cells could be observed (data not shown). After slow (–0.1°C/min) freezing in serum-containing cell culture medium (M 199) with 10% DMSO, cell viability directly after thawing was decreased to around 50% (Figure 1A; PI-negative cells). During subsequent re-culture, cell death and cell detachment progressed further, resulting in about 10% viable cells after 3 h of re-culture (Figure 1B). While control cultures formed confluent monolayers (Figure 2A), hardly any attached and intact cells were left after freezing in cell culture medium and 3 h re-culture (Figure 2B). Viability after freezing in solution 1 was only slightly higher than after freezing in cell culture medium directly after thawing (Figure 1A), but delayed cell death was markedly lower (Figure 1B) and an intact monolayer with only few detached cells was observed after 3 h of re-culture (Figure 2C). In the chloride-poor solution 2 (with balanced sodium and potassium concentrations), a solution that yielded good results in the cryopreservation of rat hepatocytes¹⁸ and in solution 3, the chloride-poor analogue of TiProtec (potassium-rich), initial viability after thawing was higher than in the other solutions (Figure 1A).

Decrease of viability during re-culture was similar to that of cells frozen in solution 1 yielding a final viability of around 50% (Figure 1B). Few cells detached and a morphologically normal monolayer was observed at the end of 3 h re-culture (Figure 2D, E).

Adherent viable cells and metabolic activity after cryopreservation

After 24 h of re-culture, the percentage of attached viable cells, as assessed by (residual) intracellular LDH (Figure 3A), was similar to the percentage of viable cells after 3 h rewarming

in all solutions (compare Figures 1B and 3A) and significantly differed between cells frozen in cell culture medium and cells frozen in solutions 2 or 3 (Figure 3A). Metabolic activity (resazurin reduction; Figure 3B) corresponded to the percentage of viable cells and was significantly higher in cells cryopreserved in solution 3 as compared to cell culture medium.

Mitochondrial integrity/membrane potential after thawing

Mitochondria were stained with two dyes: MitoTracker Green (green fluorescence), a dye

FIGURE 1. Cell viability directly after thawing and after 3 h re-culture.

Porcine aortic endothelial cell monolayers were cryopreserved ($-0.1^{\circ}\text{C}/\text{min}$) in serum-containing cell culture medium (M199), or in solution 1 (chloride-rich), solution 2 (chloride-poor, balanced Na^+/K^+ concentrations) or solution 3 (chloride-poor, potassium-rich), all supplemented with 10% DMSO. Cell viability was determined by propidium iodide (PI) staining directly after thawing (A; $n = 7$) or after 3 h of re-culture in cell culture medium (B; $n = 7$). * percentage of PI-negative cells significantly different from M199.

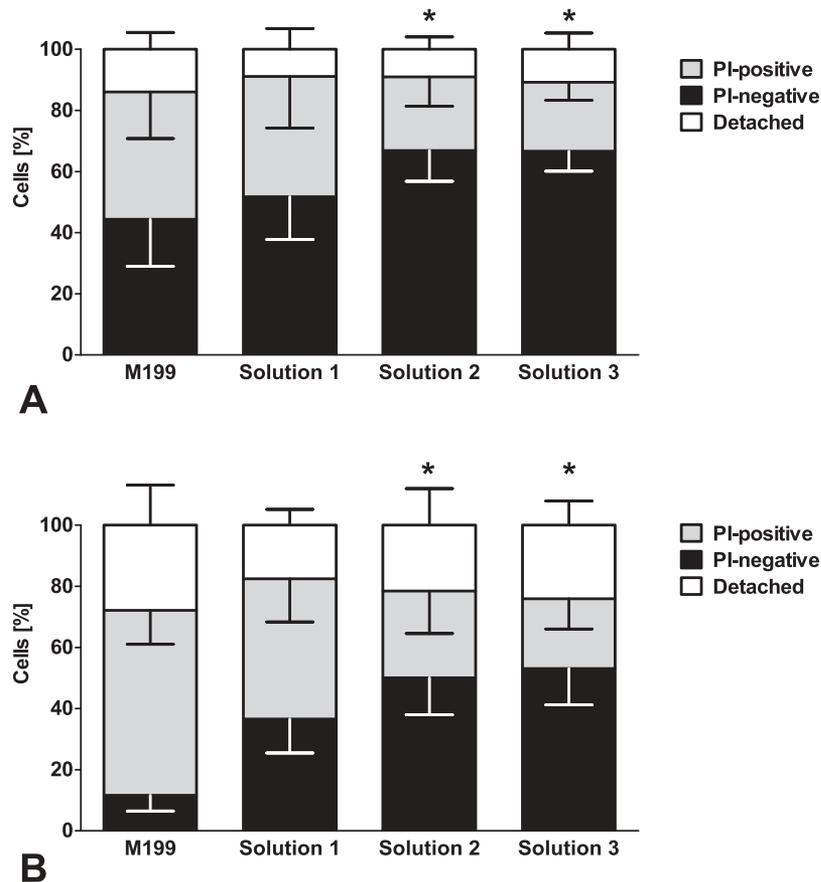
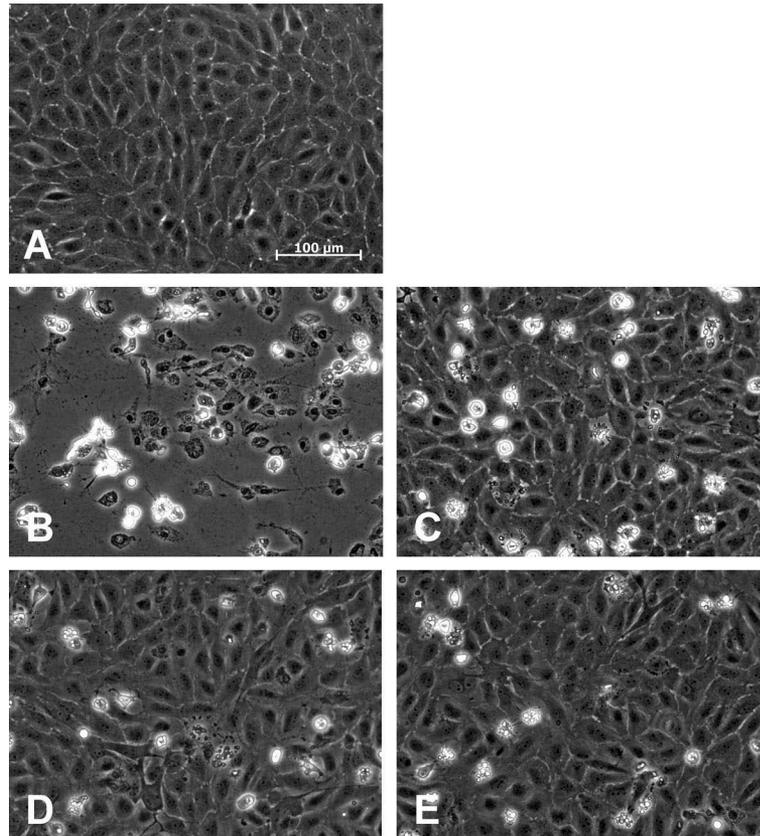


FIGURE 2. Monolayer integrity after 3 h re-culture.

Porcine aortic endothelial cell monolayers (control, A) were cryopreserved ($-0.1^{\circ}\text{C}/\text{min}$) in cell culture medium (M 199; B), solution 1 (chloride-rich; C), solution 2 (chloride-poor, balanced Na^+/K^+ concentrations; D) or solution 3 (chloride-poor, potassium-rich; E), all supplemented with 10% DMSO. Phase contrast micrographs (original magnification: 200x) were taken after 3 h of re-culture in cell culture medium.



that binds covalently and remains in the mitochondria even after the loss of mitochondrial membrane potential, and TMRM (red fluorescence), which leaks out of the mitochondria when their membrane potential decreases.

Control cells displayed long filamentous mitochondria with intact mitochondrial membrane potential (Figure 4A). Directly after thawing, mitochondrial fragmentation could be observed in all solutions (Figure 4B-E). Cells frozen in cell culture medium almost completely lost their mitochondrial membrane potential (Figure 4B) and did not recover normal mitochondrial morphology after 1 h of re-culture (Figure 4F). Of cells frozen in

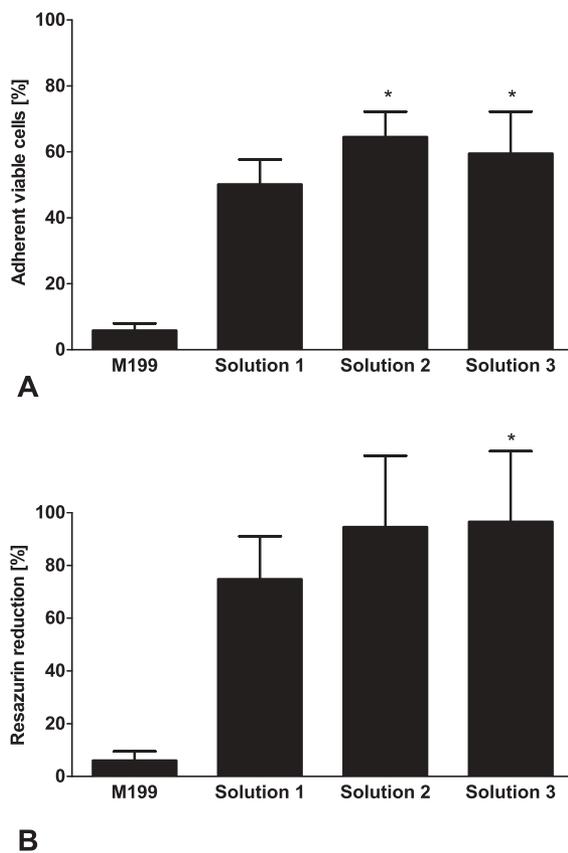
solution 1, about half of cells retained the mitochondrial membrane potential (Figure 4C) and some regained normal mitochondrial morphology after 1 h of re-culture (Figure 4G), while the proportion of cells with normal mitochondrial morphology and intact membrane potential was larger in cells frozen in solutions 2 and 3 both, directly after thawing (Figure 4D, E) and after rewarming (Figure 4H, I).

Cell proliferation after thawing

Sub-confluent cell cultures frozen in cell culture medium showed hardly any

FIGURE 3. Adherent viable cells after 24 h re-culture.

Porcine aortic endothelial cell monolayers were cryopreserved in cell culture medium (M 199), solution 1 (chloride-rich), solution 2 (chloride-poor, balanced Na^+/K^+ concentrations) or solution 3 (chloride-poor, potassium-rich), all supplemented with 10% DMSO, at a cooling rate of $-0.1^\circ\text{C}/\text{min}$. After rapid thawing and 24 h re-culture in cell culture medium, the number of adherent viable cells (as percentage of control cultures; A, $n = 6$; $P \leq .05$) and resazurin reduction (as percentage of control cultures; B, $n = 4$) was quantified. * significantly different from M199.



proliferation during 24 h re-culture after thawing ($9 \pm 10\%$ (mean \pm SD) of control cultures; Figure 5A). Cultures frozen in solution 1 displayed a significantly higher proliferation capacity than those frozen in cell culture medium (about 40% of control cultures;

Figure 5A) after re-culture; proliferation rate of cell cultures frozen in solution 2 exceeded that of cell frozen in solution 1 and reached almost 90% of the value of control cultures (Figure 5B).

Protective effect of solutions 1–3 for different cooling rates

After cryopreservation in cell culture medium and 24 h re-culture, massive cell loss/loss of proliferation ability was observed for all temperature gradients (Figure 6). The amount of living adherent cells after thawing and 24 h of re-culture was markedly improved in all new cryopreservation solutions (solutions 1–3, Figure 6); no significant difference was observed between the different variants of the new solution. Freezing at a rate of $-1^\circ\text{C}/\text{min}$ produced the best results in all solutions (Figure 6B).

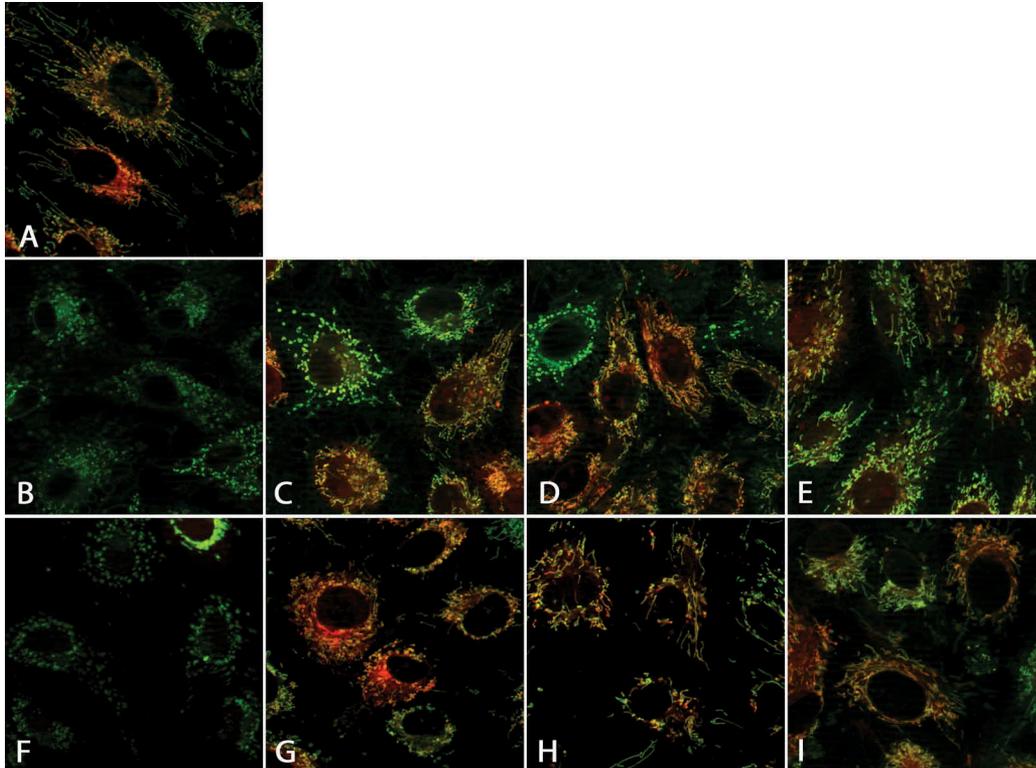
Integrity of porcine aortic segments after cryopreservation

Control segments without cryopreservation displayed a continuous endothelial monolayer and regular wall structures (Figure 7A). After cryopreservation in cell culture medium, the endothelial monolayer was either detached or widely disrupted with large gaps (Figure 7B). The endothelium of aortic segments frozen in solution 1 appeared partially detached, but less gaps in the endothelium could be observed. After freezing in solution 3, the endothelial monolayer was preserved best (Figure 7C).

DISCUSSION

Our results show that post-thaw results of endothelial monolayers and aortic segments can be greatly improved by the use of the serum- and albumin-free vascular storage solution TiProtec (and modifications thereof) as compared to standard cell culture medium (Figure 1, 2, 3, 5, 6 and 7).

FIGURE 4. Mitochondrial alterations after cryopreservation and after 1 h of re-culture. Porcine aortic endothelial cell monolayers on glass coverslips were stained with the mitochondrial dyes MitoTracker green (green, binds covalently to mitochondrial proteins) and TMRM (red, leaks out after loss of mitochondrial membrane potential; A: control cells). Afterwards, cells were cryopreserved at a cooling rate of $-0.1^{\circ}\text{C}/\text{min}$ in cell culture medium (M 199; B, F), solution 1 (chloride-rich; C, G), solution 2 (chloride-poor, balanced Na^+/K^+ ; D, H), or solution 3 (chloride-poor, potassium-rich; E, I), all supplemented with 10% DMSO. After rapid thawing (B-E) and 1 h re-culture in cell culture medium (F-I), confocal fluorescence micrographs were taken (original magnification 630x).

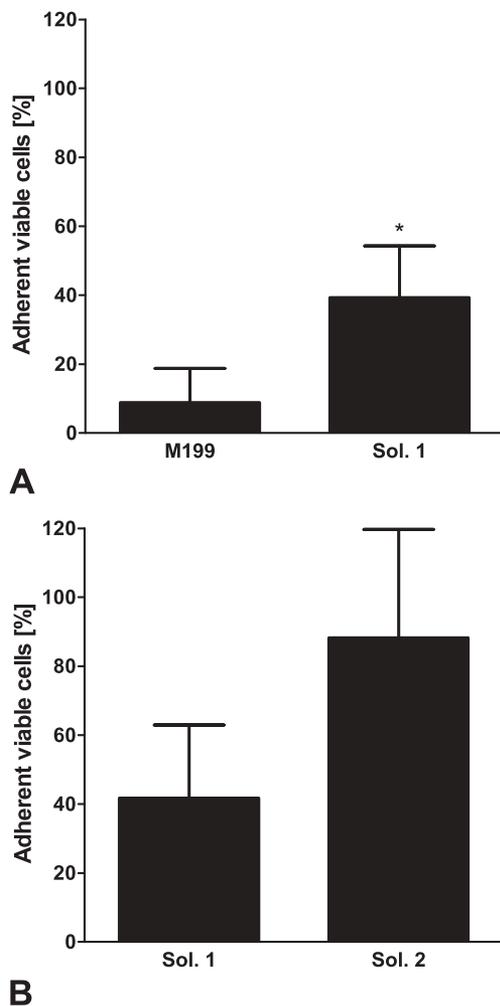


Classical view of cryoinjury

Following the classical two-factor hypothesis, cryoinjury is attributed to purely physical processes, ice crystal formation and osmotic imbalance during the freezing/thawing process. A slow cooling rate is supposed to favour freezing of extracellular water, leading to extracellular hyperosmolarity, cellular water loss and resulting in osmotic stress, while a fast decrease in temperature is regarded to promote intracellular ice crystal formation associated with mechanical disruption of the cell membrane.^{21–25} Also,

much emphasis is placed on potential cell damage caused by osmotic stress and volume excursion inflicted by the addition/removal of cryoprotectants, used in high concentrations, such as DMSO.^{25,26} These mechanisms are also considered for endothelial cells/vessels, so most approaches to optimize vascular cryoprotection are conducted along these lines.^{26–30} Little attention, however, is paid to the actual cryopreservation base solution; usually, cells and tissues are simply frozen in supplemented cell culture medium with addition of cryoprotectants.^{3–6,9,27,28,31}

FIGURE 5. Cell proliferation after thawing. Sub-confluent porcine aortic endothelial cell monolayers (24 h after sub-cultivation) were cryopreserved at a cooling rate of $-0.1^{\circ}\text{C}/\text{min}$ in cell culture medium (M 199; A), solution 1 (chloride-rich; A, B) or solution 2 (chloride-poor, balanced Na^+/K^+ ; B), all supplemented with 10% DMSO. Control cultures were kept for another 24 h at 37°C before quantification of adherent viable cells (intracellular LDH). After rapid thawing and 24 h re-culture in cell culture medium (total culture time: 48 h), cell proliferation was compared to control cultures. A: $n = 10$, B: $n = 4$; *significantly different from M199.



Cell death and proliferation delay

In addition to immediate cell death caused by the freezing/thawing process, delayed cell death

following functional impairment of the cells is discussed.²⁵ In our experiments, early cell death directly after thawing was most pronounced in standard cell culture medium (Figure 1A). Also, most delayed cell death occurred in cell culture medium (additional loss of $> 30\%$ viability; Figure 1B), while modified cold storage solutions protected against this delayed cell death ($\sim 15\%$ additional cell death during 3 h of re-culture; Figure 1B). This protective effect of the new solutions was similar in solutions 1, 2 and 3, thus being largely independent of anion and Na^+/K^+ composition.

Besides cell death, reduced proliferation rates in cryopreserved cells add to the overall cryopreservation injury. Reduced proliferation rates, at least for the first 24 h of re-culture, were described for human umbilical cord cells^{32,33} and human saphenous vein endothelial cells.³⁴ In our study, cell proliferation could be improved by use of solutions 1 or 2 compared to M199 (Figure 5).

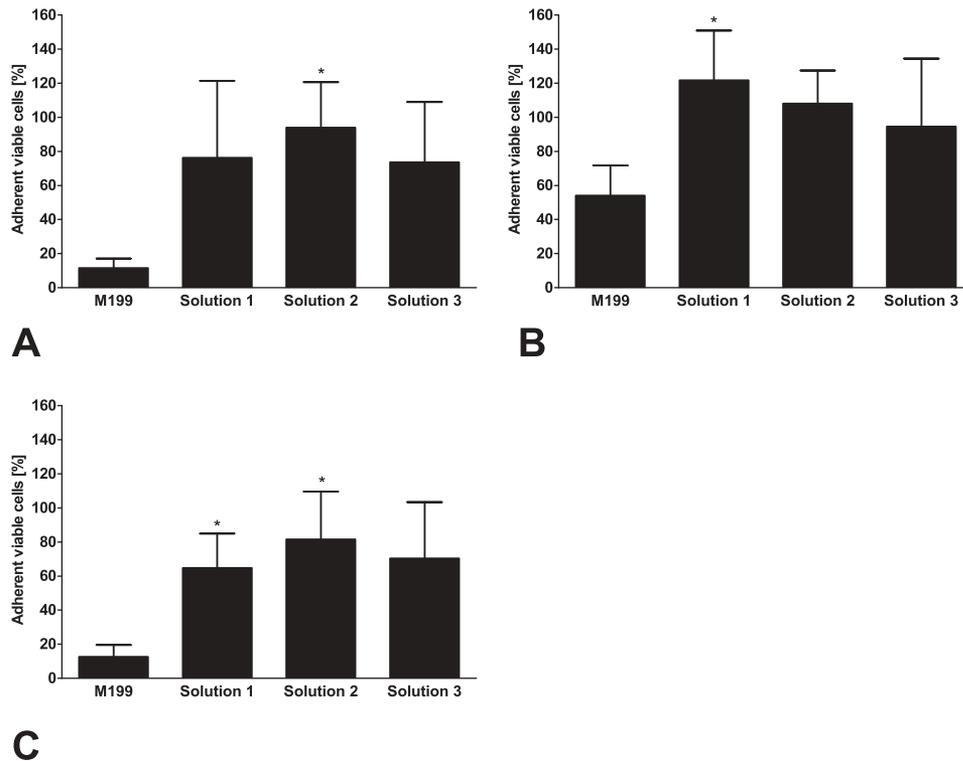
Mitochondrial impairment during cryopreservation

For cold (4°C) storage injury, loss of mitochondrial membrane potential followed by mitochondrial permeability transition was identified as main pathway for cold-induced cell injury.^{14,35,36} Loss of mitochondrial membrane potential was also described after cryopreservation, for example in sperm,³⁷⁻³⁹ mouse embryos,⁴⁰ muscle,^{41,42} hepatocytes⁴³ and in isolated mitochondria.^{44,45} Also, ultrastructural changes in mitochondria, like mitochondrial swelling and rupture have been described to occur in endothelial cells after vascular cryopreservation,^{46,47} however, little is known about mitochondrial membrane potential in this cell type. Here, almost complete loss of mitochondrial membrane potential was observed after cryopreservation in cell culture medium, while mitochondrial membrane potential was widely preserved in solutions 1, 2 and 3.

Another phenomenon described for various cell types during cold (4°C) storage is mitochondrial fragmentation, which is itself not necessarily associated with cell injury and is

FIGURE 6. Different cooling rates.

Confluent (early confluent) porcine aortic endothelial cell monolayers were cryopreserved in cell culture medium (M 199), solution 1, solution 2 (chloride-poor, balanced Na^+/K^+ concentrations) or solution 3 (chloride-poor, potassium-rich), all supplemented with 10% DMSO at cooling rates of 0.1°C/min (A; n = 7; P = .0077), 1°C/min (B; n = 7; P = .0077) and 5°C/min (C; n = 7; P = .0041). After rapid thawing and 24 h re-culture in cell culture medium, the number of adherent viable cells (as percentage of control cultures) was determined. *significantly different from M 199.



partially reversible during rewarming.^{48–50} Mitochondrial fragmentation, thought to be irreversible, was also described in cryopreserved porcine iliac arteries after rapid thawing.⁴⁶ In our study, in addition to loss of membrane potential, marked mitochondrial fragmentation after thawing was observed in all solutions, but was partially reversible during rewarming, if cells were cryopreserved in solutions 2 or 3 (Figure 4).

Influence of chloride/lactobionate

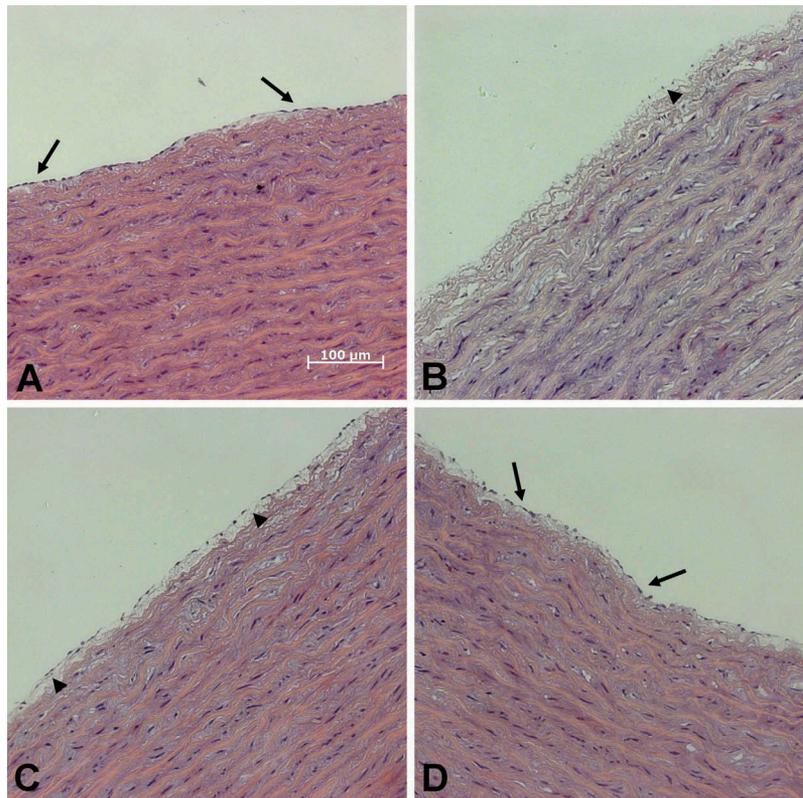
For cold (4°C) storage, porcine aortic endothelial cells prefer chloride- and potassium-rich solutions.¹⁰ Both, the high potassium concentration and a physiological extracellular

chloride concentration increased survival of aortic endothelial cells during cold storage.¹⁰ The high potassium concentration protected against the loss of the mitochondrial membrane potential.¹⁰ For the cold storage of rat renal proximal tubules, extracellular chloride has recently been shown to protect mitochondria,⁵¹ although the exact mechanism of mitochondrial protection is still unclear. In contrast to porcine aortic endothelial cells and rat renal proximal tubules, rat hepatocytes display a chloride-dependent cell injury after cold (4°C) storage in chloride-rich solutions and are therefore significantly better protected in chloride-poor solutions.^{19,20} This chloride-dependent cold-induced injury appears to involve lysosomal permeabilization.⁵² In this setting, substitution

of chloride by lactobionate might favour the maintenance of a slightly alkaline cytosolic pH (via inhibition of bicarbonate extrusion by the $\text{HCO}_3^-/\text{Cl}^-$ antiporter), thus limiting the adverse effects of released lysosomal enzymes. In contrast to the findings at 4°C, after cryopreservation, cell survival of both, rat hepatocytes¹⁸ and porcine aortic endothelial cell monolayers (Figures 1A and 3A) was higher in the chloride-poor solutions compared to their chloride-rich counterparts. Cryopreservation in chloride-poor solutions also seemed to improve protection of the endothelial layer of aortic segments (Figure 7), while high potassium concentrations had no beneficial effect (Figure 1, 2, 3, similar results in solutions 2 and 3). For

cryopreservation, in contrast to cold storage,¹⁹ this protection by the chloride-poor solutions could in part be caused by the presence of the fairly high concentration (95 mM) of the large impermeable anion lactobionate rather than by the mere absence of chloride. Lactobionate is a disaccharide derivative, and other disaccharides are known to act as cryoprotective agents in various cell types⁵³; the presence of the disaccharide sucrose in combination with DMSO has also been shown to improve functionality of cryopreserved vessels.⁵⁴ Lactobionate might stabilize the plasma membrane during cryopreservation as known for other disaccharides and/or the low concentration of chloride might avoid chloride influx via a destabilized plasma

FIGURE 7. Integrity of porcine aorta segments after thawing. Segments of porcine aortae (control; A) were cryopreserved in cell culture medium (B), solution 1 (C) or solution 3 (chloride-poor; D), all supplemented with 10% DMSO. After quick thawing in a 37°C water bath segments were fixed. Sections were stained with haematoxylin and eosin. Arrows: intact endothelial monolayer; arrowheads: detached endothelium.



membrane/fluid-to-gel phase transition during freezing/thawing.⁵⁵ Chloride fluxes in the frozen state, in contrast, are unlikely. Although the mechanism of lactobionate protection during cryopreservation is not resolved yet, it is interesting that for porcine aortic endothelial cells lactobionate-rich, chloride-poor solutions are favourable during cryopreservation while they are unfavourable during hypothermic storage (where, however, ion fluxes can occur during the whole storage period). Further experiments will have to show whether this is a general principle for cryopreservation (irrespective of the need for high or low chloride concentrations during hypothermic storage) or whether other cell types react differently.

Serum/albumin in cryopreservation

Addition of albumin or serum is generally thought to improve cryopreservation results.^{5,56} Also, since cryopreservation is often performed in supplemented cell culture media, high concentrations of serum are often already present in the cryopreservation solution.^{6,46,57} However, especially for the use in tissue banking, serum-free cryopreservation is desirable, as serum is *i)* undefined and *ii)* of animal origin. Here, better results were achieved in serum-free cryopreservation solutions than in completed M 199 with 20% foetal calf serum (Figure 2, 3, 6).

Influence of different temperature gradients

The chosen freezing protocols with a short temperature dip to initiate crystallization and absorb the heat release, which result in a more or less linear decrease of sample temperature, showed good results in other cell types.^{58,59} Optimum cooling rates not only differ between different cell types, but also between cells in suspension and attached cells.^{60,61} Cells in suspension seem less affected by changes of cooling rates than attached cells, which show increasing cryopreservation damage with increasing cooling rates and meet their optimum cooling rates well below the standard temperature slope of $-1^{\circ}\text{C}/\text{min}$.^{60,61} We here compared three

different cooling rates (0.1°C , 1°C and $5^{\circ}\text{C}/\text{min}$) and observed better results for solutions 1–3 than for cell culture medium with all three. Surprisingly, in contrast to previous publications on immortalized human endothelial cell monolayers⁶⁰ and corneal keratinocytes,⁶² best results for porcine aortic endothelial cell monolayers were not achieved with the slowest cooling rate ($0.1^{\circ}\text{C}/\text{min}$; Figure 6A), but with a standard cooling rate of $-1^{\circ}\text{C}/\text{min}$ (Figure 6B). Von Bomhard et al. also produced good results with endothelial cell suspensions in a modified TiProtec solution in vitrification experiments (plunging in liquid nitrogen).⁶³

Monolayer model

In this study, we used endothelial cell monolayers as 2D-model of vascular endothelium that features cell-cell-contact between endothelial cells. Compared to cell suspensions, monolayers seem more susceptible to cryo-injury, especially at higher cooling rates,⁶¹ which might be caused by intracellular ice crystal formation progressing via gap junctions.^{61,64} However, in cryopreserved vessels, more variables, like different requirements of endothelial cells and smooth muscle cells²⁶ and fracturing of the vascular tissue after cryopreservation^{65,66} have also to be taken into account. As a simplified 3D-model, we here used aortic segments. First results indicate that the modified solutions also have a protective effect on endothelium and wall integrity in this compound tissue (Figure 7). Clearly, further experiments with other vascular cells, with endothelial cells of other vascular provinces (differing in chloride conductivity) and with other vessel types will be required in the future.

Outlook

In a next step, the results of our experiments in endothelial monolayers and aortic segments have to be verified in 3D-models such as tissue-engineered vascular constructs and complete vessels, especially in small vessels in which endothelial integrity is particularly important.

MATERIALS & METHODS

Chemicals and cryopreservation solutions

Freezing solutions are based on solution 8 of Wille et al.¹⁰, which is potassium- and chloride-rich and contains iron chelators (solution 1, Table 1; commercially available (with minor modifications) as the vascular preservation solution TiProtec[®]), since these components showed to be protective in cold storage of porcine aortic segments. Solution 2 is a chloride-poor variant with balanced Na⁺/K⁺ concentrations, as used earlier for cryopreservation of rat hepatocytes.¹⁸ For direct comparison to TiProtec in terms of chloride concentration, solution 3 was used, which resembles solution 1 with respect to cation composition (high potassium concentration) but is chloride-poor (Table 1). *N*-acetylhistidine and the iron chelator LK 614 were kindly provided by Dr. F. Köhler Chemie; all solutions (composition see Table 1) were prepared in our laboratory. All other chemicals were obtained from Sigma-Aldrich or Merck KGaA.

Cell culture

Porcine aortic endothelial cells were isolated as described by Peters et al. by gently scraping the endothelial cells off the intima of porcine aortae.⁶⁷ Cells were cultured in medium 199 (M 199) with Earle's salt, supplemented with 100 IU/mL penicillin G, 100 µg/mL streptomycin, and 20% (*vol/vol*) fetal bovine serum. Subcultures were obtained by trypsinization (0.25% trypsin). For the experiments cells were split 1:3 and seeded onto collagen-coated six-well-plates (Sarstedt, #83.3920) or, for fluorescence microscopy, onto collagen-coated 28 mm glass coverslips (Thermo Scientific). The cells were used for experiments 2 days after subcultivation.

Experimental procedure – freezing/thawing

At the beginning of the experiments, cell cultures were washed three times with Hanks' balanced salt solution (HBSS; 37°C), covered with supplemented cell culture medium, TiProtec or modified solutions (Table 1; all

supplemented with 10% DMSO) at room temperature, and subsequently incubated at 4°C for 10 min to allow DMSO to enter the cells. Cell cultures were then transferred to the pre-cooled (4°C) cooling chamber of a controlled-rate freezer and cooled at a linear temperature gradient (−0.1°C/min for most experiments, −1°C/min and −5°C/min in additional series) to −4°C sample temperature. Then a short, rapid temperature drop to −40°C chamber temperature and immediately back to the approximate sample temperature was inserted to initialize crystallization, followed by linear decrease to −80°C at the initial cooling rate. For the slowest protocol (−0.1°C/min), cooling rate was increased to −1°C/min at a sample temperature of −20°C. After 18–24 h storage at −80°C the monolayers were rapidly thawed in a shaking water bath (3 min, 37°C) and re-cultured in supplemented cell culture medium (M 199) for 1 to 24 hours.

Assessment of viability

Directly after thawing and after 3 h of re-culture, cells were stained with propidium iodide (PI; 5 µg/mL). Phase contrast micrographs and red fluorescence micrographs were taken using a Zeiss Observer.Z1 (Zeiss; $\lambda_{exc} = 546 \pm 6 \text{ nm}$ / $\lambda_{em} \geq 590 \text{ nm}$). Dead cells (red fluorescent nuclei) and total cells were counted in nine fields of vision and viability was calculated as percentage of non-frozen control cells, thus taking into account the cell loss caused by cell detachment.

Determination of adherent viable cells

After 24 h of re-culture, adherent viable cells were quantified by assessment of (residual) intracellular lactate dehydrogenase (LDH) as described previously.²⁰

Metabolic activity

Cells were washed with HBSS, and 40 µM resazurin in warm HBSS + 10 mM D-glucose was added to the wells. Reductive conversion of resazurin to resorufin was followed in a plate

TABLE 1. Composition of cryopreservation solutions.

	M 199 ^a	Solution 1	Solution 2	Solution 3
Cl ⁻	125.8	103.1	8.1	8.1
α -Ketoglutarate	-	2	2	2
Aspartate	-	5	5	5
Lactobionate	-	-	95	95
H ₂ PO ₄ ⁻	0.9	1.0	1.0	1.0
SO ₄ ²⁻	0.8	-	-	-
HCO ₃ ⁻	26.2	-	-	-
NO ₃ ⁻	4 x 10 ⁻³	-	-	-
Na ⁺	144.1	16	60	16
K ⁺	5.4	93	49	93
Mg ²⁺	0.8	8	8	8
Ca ²⁺	1.80	0.05	0.05	0.05
<i>N</i> -Acetylhistidine	-	30	30	30
Glycine	0.7	10	10	10
Alanine	0.3	5	5	5
Tryptophan	0.1	2	2	2
Sucrose	-	20	20	20
Glucose	5.6	10	10	10
Deferoxamine	-	0.1	0.1	0.1
LK 614	-	0.02	0.02	0.02
pH	7.4	7.0	7.0	7.0
Calculated osmolality (mosm/L)	n.d.	305	305	305

All solutions were supplemented with 10% DMSO for cryopreservation.

^aM 199 additionally contains amino acids, vitamins and trace elements and was supplemented with 20% FCS, 100 IU/mL penicillin G and 100 µg/mL streptomycin.

reader (FluoStar Optima, BMG Labtec) at $\lambda_{exc.} = 560 \pm 10$ nm and $\lambda_{em.} \geq 590$ nm at 37°C for 10 min and conversion rate was calculated as percentage of that of control cultures.

Proliferation capacity

Sub-confluent endothelial cell cultures (24 h after subcultivation) were frozen at $-0.1^\circ\text{C}/\text{min}$ in M199, solution 1 or solution 2, each with 10% DMSO. Control cultures were cultured for another 24 h at 37°C (48 h total culture time after subcultivation) before lysis and determination of intracellular LDH. After 18–24 h storage at -80°C , frozen monolayers were rapidly thawed in a shaking water bath (3 min, 37°C) and re-cultured in supplemented cell culture medium (M 199) for 24 hours. Adherent viable cell density was then compared to control cultures (48 h after subcultivation) to compare proliferation rates.

Fluorescence microscopy of the mitochondria

For determination of mitochondrial integrity by fluorescence microscopy, cells were seeded onto collagen-coated glass coverslips in six-well plates. Before cryopreservation, cells were loaded for 40 min with 500 nM MitoTracker Green (molecular probes, #M7514) and 20 min with 200 nM tetramethyl rhodamine methyl ester (TMRM) in HBSS at 37°C. For TMRM, a maintenance concentration of 20 nM was used after loading. After thawing, the cryopreservation solutions were exchanged for Krebs-Henseleit buffer with 10 mM D-glucose and 20 nM TMRM. Fluorescence was assessed by confocal fluorescence microscopy using a Zeiss Observer.Z1 with ApoTome module (Zeiss) with filter sets for $\lambda_{exc} = 546 \pm 6$ nm/ $\lambda_{em} \geq 590$ nm (TMRM) and $\lambda_{exc} = 470 \pm 20$ nm/ $\lambda_{em} = 525 \pm 25$ nm (MitoTracker Green).

Studies in aortic segments

Porcine aortic segments were frozen in cell culture medium, solution 1 or solution 3 (50 mL, all supplemented with 10% DMSO) in 100-mL freezing bags at $-1^{\circ}\text{C}/\text{min}$. After thawing (5 min in a water bath at 37°C), segments were fixed in formalin, embedded in paraffin blocks, cut and stained with haematoxylin and eosin.

Statistical analysis

Cell culture experiments were performed in triplicates 4–10 times (see respective figure legends). The Friedman test with post-hoc Dunn's multiple comparison test was used to compare M199 with solutions 1–3 and solution 1 with solution 3. To limit the influence of inter-experiment variability, only sets from the same cell isolation were compared. $P \leq .05$ was considered statistically significant.

DISCLOSURE OF INTEREST

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