AMERICAN JOURNAL OF PHYSIOLOGY
LUNG CELLULAR AND MOLECULAR PHYSIOLOGY

Am J Physiol Lung Cell Mol Physiol 321: L1023–L1035, 2021.
First published October 13, 2021; doi:10.1152/ajplung.00076.2021

RESEARCH ARTICLE

Translational Physiology

Optimization of long-term cold storage of rat precision-cut lung slices with a tissue preservation solution

Jonas Tigges,[1] Florian Eggerbauer,[2] Franz Worek,[1] Horst Thiermann,[1] Ursula Rauen,[3] and Timo Wille[1]

[1]*Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany;* [2]*Walther Straub Institute of Pharmacology and Toxicology, Munich, Germany; and* [3]*Institute of Physiological Chemistry, University Hospital, Essen, Germany*

Abstract

Precision-cut lung slices (PCLS) are used as ex vivo model of the lung to fill the gap between in vitro and in vivo experiments. To allow optimal utilization of PCLS, possibilities to prolong slice viability via cold storage using optimized storage solutions were evaluated. Rat PCLS were cold stored in DMEM/F-12 or two different preservation solutions for up to 28 days at 4°C. After rewarming in DMEM/F-12, metabolic activity, live/dead staining, and mitochondrial membrane potential was assessed to analyze overall tissue viability. Single-cell suspensions were prepared and proportions of $CD45^+$, $EpCAM^+$, $CD31^+$, and $CD90^+$ cells were analyzed. As functional parameters, TNF-α expression was analyzed to detect inflammatory activity and bronchoconstriction was evaluated after acetylcholine stimulus. After 14 days of cold storage, viability and mitochondrial membrane potential were significantly better preserved after storage in *solution 1* (potassium chloride rich) and *solution 2* (potassium- and lactobionate-rich analog) compared with DMEM/F-12. Analysis of cell populations revealed efficient preservation of $EpCAM^+$, $CD31^+$, and $CD90^+$ cells. Proportion of $CD45^+$ cells decreased during cold storage but was better preserved by both modified solutions than by DMEM/F-12. PCLS stored in *solution 1* responded substantially longer to inflammatory stimulation than those stored in DMEM/F-12 or *solution 2*. Analysis of bronchoconstriction revealed total loss of function after 14 days of storage in DMEM/F-12 but, in contrast, a good response in PCLS stored in the optimized solutions. An improved base solution with a high potassium chloride concentration optimizes cold storage of PCLS and allows shipment between laboratories and stockpiling of tissue samples.

cold storage; PCLS; 3R; tissue preservation

INTRODUCTION

Precision-cut lung slices (PCLS) are a widely used tool in medical research to study lung biology in an ex vivo tissue system (1). Compared with cell cultures, PCLS exhibit several advantages. Cell cultures usually involve a limited number of different cell types, whereas PCLS consist of all resident lung cells in their natural spatial relationship representing the original complexity of the organ. Cells are embedded in their natural structural arrangement, thus yielding an optimal prediction system to study the effects on lung architecture and physiological organ function including cell-cell interactions and matrix effects (2). PCLS offer the opportunity to study structural and physiological alterations upon treatment in specific airway sections or distal lung, which is of increasing interest for the use in preclinical validation. Thereby, biochemical analysis methods such as viability assays, detection of inflammatory markers, RNA, and protein analysis, as well as microscopical investigations can be applied (3–5). In addition, PCLS are a promising tool not only for the development of novel treatment strategies for airway diseases like asthma, chronic obstructive pulmonary disease (COPD), or inflammation-mediated pulmonary damage but also for the development of medical countermeasures against the cholinergic crisis after exposure to organophosphorus pesticides or nerve agents (1, 6, 7). PCLS contain responsive airways, enabling the quantification of airway constriction after a pharmacological stimulus (6, 8). Addition of the neurotransmitter acetylcholine (ACh) to PCLS medium results in constriction of airway smooth muscle cells and decrease of the airway cross-section area. This bronchoconstriction is spontaneously reversible due to lung-resident active acetylcholinesterase (6, 9). Due to the development of specialized tissue-slicers, it is possible to provide a large number of highly standardized tissue samples with low tissue damage within a short time (10). Numerous PCLS (100–150) can be prepared from one rat lung, thus reducing the number of animals needed for in vivo experiments which is in line with the 3 "R" approach (11). As human lung tissue specimens are rare and samples of diseased patients are even more precious, an optimized storage protocol to process high numbers of PCLS is highly desirable. Storage of

Correspondence: T. Wille (TimoWille@bundeswehr.org).
Submitted 17 February 2021 / Revised 29 September 2021 / Accepted 11 October 2021

PCLS is standardized for cell culture conditions (37°C, 5% CO_2 submersed in a standard cell culture medium) as the required infrastructure is available in most laboratories. PCLS can be maintained at 37°C without significant loss of viability for up to 14 days if the medium is frequently changed (12). Further improvements have been made by modification of the culture medium with supplements like insulin (13). However, the transport on a research campus or shipment to other laboratories is difficult under cell culture conditions. To overcome this issue and to provide tissue samples for cooperation partners at other locations, shipment of PCLS with an easy to standardize storage protocol is highly desirable. In addition, optimized storage of PCLS allows stockpiling of tissue samples for on-demand experiments, minimizes the required number of animals, and maximizes the yield of PCLS from human lung samples.

Cryopreservation of PCLS allows a virtually unlimited storage duration and rewarming on demand (14–16). Yet, freezing of biological samples requires special equipment to control the freezing process, and air shipment using dry ice for temperature control is challenging due to strict regulations of the International Air Transport Association (IATA). Freezing may result in loss of intracellular water, ice crystal formation, and destruction of the cellular architecture, which calls for the use of cryoprotectants that can cause cellular lesions themselves (17–19). Although PCLS seem to withstand freeze-thaw procedures in terms of viability and airway constriction (14), a significant decrease in metabolic activity has been detected after cryopreservation (16, 20). A more "gentle" way to preserve tissue slices is long-term hypothermic (cold) storage at 4°C, which does not necessitate special devices and was already optimized for use in precision cut liver and kidney slices (21–23), but to the best of our knowledge has not been investigated yet for PCLS storage. Recently developed cold storage solutions have been successfully used for the preservation of human lung epithelial cells (A549), hepatocytes, and tissues such as blood vessels and striated muscles (24–26).

Thus, we here set out to analyze the effects of long-term cold storage in standard culture medium and two optimized preservation solutions (either with high potassium chloride concentrations or a chloride-poor, lactobionate-rich analog) on cellular viability, mitochondrial membrane potential, cell composition, inflammatory activation, and bronchoconstriction in rat PCLS.

METHODS

Chemicals

For Tyrode-buffer preparation, 2.68 mmol/L KCl (Carl Roth, Karlsruhe, Germany), 1.05 mmol/L $MgCl_2 \cdot 6H_2O$ (Sigma-Aldrich, St. Louis, MO), 0.42 mmol/L $NaH_2PO_4 \cdot 2H_2O$ (Merck KGaA, Darmstadt, Germany), 137 mmol/L NaCl (Carl Roth, Germany), 1.8 mmol/L $CaCl_2 \cdot 2H_2O$ (Carl Roth, Germany), 22 mmol/L $NaHCO_3$ (Carl Roth, Germany), and 5.5 mmol/L glucose monohydrate (Merck KGaA, Germany) were dissolved in double distilled water and cooled on ice until use for PCLS preparation. Afterward, pH was adjusted to 7.4 by carbogen gassing. Assays were performed in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (1:1) without phenol red and L-glutamine (DMEM/F-12; Sigma-Aldrich, St. Louis, MO) supplemented with 1% penicillin-streptomycin (Sigma-Aldrich) and 0.1% gentamycin (Thermo Fisher, Waltham, MA). The low melting point agarose was purchased from Sigma-Aldrich. The cold storage solutions 1 and 2 were prepared in the Institute of Physiological Chemistry, Essen, Germany (Table 1). The supplement LK 614 (*N*-hydroxy-3,4-dimethoxy-*N*-methylbenzamide) was kindly provided by Dr. Franz Köhler Chemie, Bensheim, Germany, and deferoxamine mesylate was purchased from Novartis Pharma (Basel, Switzerland). For bronchoconstriction experiments, a stock solution (0.1 mol/L in DMEM/F-12) of ACh (Sigma-Aldrich) was prepared and stored at −80°C. The working solution (50 μmol/L in DMEM/F-12, final concentration 0.5 μmol/L) was freshly prepared at the day of the experiment.

Animals

For preparation of PCLS, male Wistar rats were purchased from Charles River Laboratories (Sulzfeld, Germany) and were kept in a standard animal housing unit providing an automated 12-h light/dark cycle and air conditioning. Animals were fed with a standard diet and drinking water ad libitum. Upon arrival in the animal housing, rats were kept for at least seven days before using them for PCLS preparation to allow a proper acclimatization (final weight 400–500 g). All experiments were in accordance with the German Animal Welfare Act of 18th May 2006 (BGB1, I S. 1206, 1313) and the European Parliament and Council Directive of 22nd September 2010 (2010/63/EU).

PCLS Preparation

For preparation of PCLS, a procedure as described by Herbert et al. (6) with some modifications was applied. Briefly, rats were anesthetized by intraperitoneal injection of a mixture of 75 mg/kg ketamine (Ketavet 100 mg/mL, zoetis Deutschland GmbH, Berlin, Germany) and 10 mg/kg

Table 1. *Composition of the applied preservation solutions and DMEM/F-12 culture medium*

	DMEM/F-12	*Solution 1*	*Solution 2*
Cl^-	126	103.1	8.1
Lactobionate			95
Na^+	133	16	16
K^+	0.4	93	93
$H_2PO_4^-$		1	1
SO_4^{2-}	0.4		
Mg^{2+}	1	8	8
Ca^{2+}	2.22	0.05	0.05
Glycine	0.25	10	10
Alanine	0.05	5	5
α-Ketoglutarate		2	2
Aspartate	0.05	5	5
N-acetylhistidine	0.15	30	30
Tryptophan	0.05	2	2
Sucrose		20	20
Glucose	17.5	10	10
HEPES buffer	15		
pH	7.0–7.6	7.0	7.0
Deferoxamine	(0.1)	(0.1)	(0.1)
LK 614	(0.02)	(0.02)	(0.02)

The concentrations of all compounds are given in mmol/L. Brackets mark concentrations of optionally added compounds.

xylazine (Xylasel 20 mg/mL; Selectavet Dr. Otto Fischer GmbH, Weyarn-Holzolling, Germany). The abdominal cavity was opened and the animals were euthanized by exsanguination. Afterward, the thorax was opened and the trachea was cannulated. Low melting point agarose (1.5% in DMEM/F-12) was gently heated until boiling and kept at 37°C. About 20 mL of liquid agarose were filled into the lung in situ, using a 20-mL syringe until the lung lobules were entirely filled. Afterward, the lung was removed from the thoracic cavity and cooled on ice for 10 min, followed by additional storage for 20 min in 4°C precooled DMEM/F-12 to allow complete solidification of the agarose solution. Subsequently, the lung lobes were separated and tissue cylinders with a diameter of 8 mm were generated using a biopsy punch. The cylinders were sliced into 250–300 μm thick PCLS using a Krumdieck Tissue Slicer (Alabama Research and Development, Munford, AL). Freshly prepared, ice-cold Tyrode-buffer (pH 7.4) was used as slicing medium. Slicing medium was changed after 5 cores and PCLS were collected in precooled DMEM/F-12. Afterward, PCLS were placed in an incubator (HeraCell 240i; Thermo Fisher Scientific, Waltham, MA) at 37°C and 5% CO_2 on a shaker (60 rpm) to partly wash out the agarose and to remove cellular debris. Medium was exchanged every 30 min for 1.5 h and afterward every 60 min for another 2 h.

Storage of PCLS and Preparation before Experimental Use

For the evaluation of viability after storage at 37°C, each PCLS was transferred into one well of a 24-well plate filled with 500 μL prewarmed DMEM/F-12. PCLS were incubated for up to 14 days without medium exchange to enable comparison with cold-stored slices. For cold storage, a 24-well plate was filled with DMEM/F-12, *solution 1*, or *solution 2*, each either with or without chelator additives (Table 1). Afterward, slices were transferred into the plates (one slice per well) and stored for 3–28 days at 4°C in the different storage solutions. As control, the following assays were also performed with unstored PCLS, held at 37°C for no longer than 24 h until the start of the experiment. At the end of the cold storage period, PCLS were transferred into 500 μL cold DMEM/F-12 and rewarmed to 37°C in the incubator on a shaker for 3 h (60 rpm) similar to the wash process directly after preparation.

Metabolic Activity in PCLS

To detect the effects of cold storage on overall slice metabolic activity, an Alamar Blue assay (Invitrogen, Carlsbad, CA) was performed. The Alamar Blue assay has been successfully used in PCLS (27) and is based on the reduction of resazurin to the fluorescent dye resorufin by metabolically active cells, and therefore serves as marker for cellular viability. One PCLS per solution was transferred into DMEM/F-12 and the Alamar Blue reagent was added in a relation of 1:10 according to the manual. After 2 h of incubation at 37°C, PCLS supernatant was transferred into a 96-well plate in duplicates. Fluorescence intensity was detected using a plate-reading photometer (Tecan Infinite 220 PRO, Tecan Group Ltd., Mennedorf, Switzerland) at excitation wavelength of 560 nm and an emission wavelength of 590 nm. Background fluorescence was subtracted and the signal intensity of cold-stored and rewarmed PCLS was compared with freshly prepared slices. For evaluation of the metabolic activity during cold storage, the same procedure was also applied to PCLS in the modified storage solutions at 4°C.

Calcein AM/Ethidium Homodimer-1 Staining

For qualitative evaluation of cold storage-induced cell death, a calcein acetoxymethyl/ethidium homodimer-1 (calcein AM/EthD-1) staining kit (Invitrogen, Karlsruhe, Germany) and subsequent evaluation by confocal microscopy was applied. Directly after preparation (*day 0*) as well as after cold storage in the modified solutions or DMEM/F-12 (*day 14*) and subsequent rewarming for 3 h, PCLS were stained with 4 μmol/L calcein AM (enzymatic conversion to the green, fluorescent dye calcein by living cells) and 4 μmol/L EthD-1 (cell impermeable dye that generates a red fluorescent signal at the cell nuclei) in DMEM/F-12 for 45 min. Afterward, lung slices were washed in phosphate buffered saline (PBS) and weighted with steel wires to prevent floating during investigation with a confocal microscope (Leica DMi-8; Leica Microsystems, Wetzlar, Germany) using a ×10 objective [excitation/emission maxima: 494/517 nm (calcein AM) and 528/617 nm (EthD-1)]. Z-stacks were generated for qualitative evaluation of PCLS viability and three-dimensional (3-D) images of PCLS were prepared by channel overlay with surface rendering applying equal settings to all images.

Analysis of Mitochondrial Membrane Potential

To analyze the effects of cold storage on mitochondria in PCLS, a tetramethylrhodamine methyl ester (TMRM) Assay Kit (Abcam, Cambridge, UK) in combination with confocal microscopy analysis was applied. TMRM is a cell permeable dye that accumulates in active mitochondria of viable cells and is used to detect mitochondrial membrane potential without the need for tissue fixation. Directly after preparation or after storage at 4°C, PCLS were transferred into DMEM/F-12 and rewarmed to 37°C for 3 h. TMRM staining was performed as indicated in the manufacturer's instructions. Staining solutions were prepared using serum-free DMEM/F-12 medium. PCLS were examined with an inverted confocal fluorescence microscope (Zeiss LSM 710, Carl Zeiss AG, Oberkochen, Germany), EC Plan-Neofluar ×10/0.30 M27 objective, 90-μm pinhole, in ×100 magnification using an excitation/emission wavelength of 548/573 nm. Two representative regions per slice were examined and Z-Stack images were generated. Mean fluorescence intensity of each image was calculated using ImageJ software (version 1.52a, National Institutes of Health, Bethesda, MD). Mean fluorescence intensity after storage is expressed in relation to that of unstored PCLS.

Preparation of Single-Cell Suspensions and Cell Type Identification

After storage for the indicated time periods and subsequent rewarming, 20 PCLS per solution were combined and incubated for 30 min in a digestion solution, containing 50 U/mL Dispase II (Corning, New York) in DMEM/F-12 medium. Single cells were prepared by passing the suspension through a cut 1-mL tip and afterward through a 100-μm cell

strainer. The strainer was washed with DMEM/F-12 containing 0.04 mg/mL DNAse I (Applichem, Darmstadt, Germany). Then, cells were passed through a 40-μm strainer and counted using a Neubauer counting chamber. Next, 500,000 cells per storage condition were blocked in PBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) and afterward stained for 1 h with either mouse anti-rat CD45-FITC (Clone OX-1; 1:100) and mouse anti-rat EpCAM/TROP1-AF594 (Clone EGP40/1110; 1:100) or mouse anti-rat CD31/PECAM-1-AF647 (Clone TLD-3A12; 1:100) and mouse anti-rat CD90/Thy1-DL550 (Clone OX-7; 1:100) or respective isotype controls (all from Novus Biologicals, Centennial, CO). Afterward, cells were washed in PBS containing 1% BSA and fixed in 4% paraformaldehyde (PFA) for 15 min. After another washing step, cells were resuspended in PBS with 1% BSA and stored at 4°C until analysis using an Amnis ImageStreamX multispectral imaging flow cytometer (Amnis Corporation, Seattle, WA). Single cells in the focus of the attached microscope were gated and thereof, percentage of positively stained cells was calculated for each marker regarding autofluorescence and isotype controls (Amnis IDEAS version 6.2). Staining was visually confirmed by evaluation of microscopic cell images (×40 objective).

Inflammatory Cytokine Expression

To evaluate the functionality of inflammatory cells after cold storage, PCLS were rewarmed for 3 h and subsequently incubated in the presence or absence of 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) in DMEM/F-12 for 24 h. Afterward, PCLS supernatant was removed, snap-frozen in liquid nitrogen and stored at −80°C. Expression of the inflammatory cytokine tumor necrosis factor-α (TNF-α) was quantified using an enzyme-linked immunosorbent assay kit (ELISA; DuoSet ELISA, R&D, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions.

Bronchoconstriction

PCLS were rewarmed for 3 h in DMEM/F-12 and weighted with a triangular steel wire in a 24-well plate. The prepared plate was transferred to the microplate-holder of an inverted microscope (Axio Observer D1, Carl Zeiss AG). Using the AxioCam HSm camera (Carl Zeiss AG) and the AxioVision software (Version 4.8.2.0, Carl Zeiss AG), airway cross-sections were observed for signs of vitality such as beating cilia on the airway epithelium and spontaneous muscle contractions. Before ACh application, the initial airway area was calculated. If slices showed spontaneous muscle tonus, the picture of the widest opening of the airway was used. Two minutes after ACh application (0.5 μmol/L final concentration), a second picture was taken to evaluate time-dependent airway constriction [Fig. 1; for a detailed discussion on bronchoconstriction and alternatives to ACh stimulation the reader is referred to the study by Herbert et al. (6)]. Afterward, airway area was measured using the AxioVision software. Bronchoconstriction of unstored slices was set as 100% and airway constriction of all cold-stored PCLS was related to that value. During bronchoconstriction experiments, airways were routinely inspected for the presence or absence of ciliary beating by video microscopy, which serves as additional marker for the functional status of PCLS.

Data Analysis

Data are presented as means ± SE. Statistical analyses were performed using GraphPad Prism Version 5.04 (GraphPad Software, San Diego, CA). Statistics were determined by two-way ANOVA with Bonferroni multiple comparison test. Viability of PCLS stored at 37°C was analyzed by one-way ANOVA with Dunnett's multiple comparison test. Detailed *n* numbers used for each experiment can be found in the figure legends. $P < 0.05$ was considered to be statistically significant.

RESULTS

Viability after Storage

To determine storage-dependent effects on PCLS viability, slices were stored for up to 14 days in the incubator at 37°C and 5% CO_2 without DMEM/F-12 medium change. Afterward, an Alamar Blue assay, based on the reduction of resazurin to the fluorescent dye resorufin, was performed, revealing a time-dependent decline in viability, which was significantly different from day ≥7 (68 ± 6%; $P < 0.05$ vs. *day 0*) and further decreased until *day 10* (60 ± 11%) with an almost complete loss on *day 14* (10 ± 7%). During cold storage, metabolic activity was significantly decreased in DMEM/F-12, *solution 1*, and *solution 2* (Table 1) with or without the iron chelators deferoxamine and LK 614 and ranged between 4 ± 1% and 6 ± 1% ($P < 0.001$ vs. incubator stored PCLS).

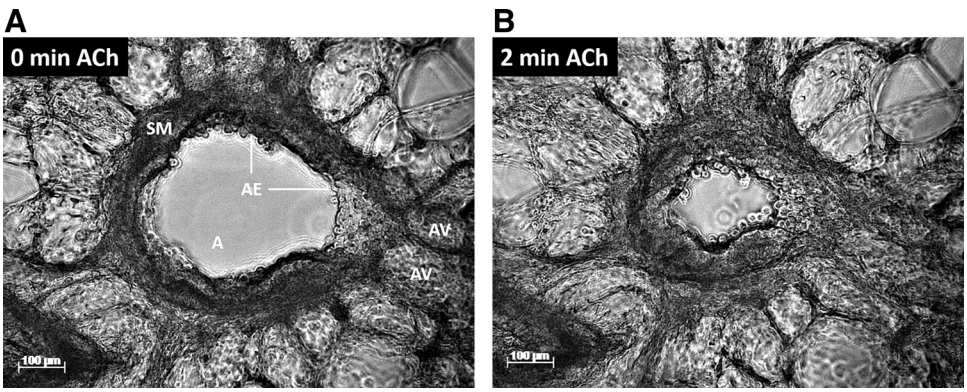

Figure 1. Precision-cut lung slices (PCLS) airway cross sections before (*A*) and 2 min after (*B*) stimulation with acetylcholine (ACh) (×200 magnification). A, airway area; AE, airway epithelium; AV, alveolar spaces; SM, smooth muscle. Scale bar, 100 μm.

To extend storage duration of PCLS without medium change, PCLS were cold-stored in DMEM/F-12 or two specifically designed preservation solutions (Table 1) with and without iron chelators at 4°C for 3–28 days and were subsequently rewarmed in DMEM/F-12 for 3 h. After 3 days of cold storage and rewarming to 37°C, viability decreased to 70%–80% with all tested solutions compared with unstored control PCLS (Fig. 2). After 7 days, viability of PCLS significantly decreased at all tested conditions ($P < 0.05$ vs. unstored). The viability of DMEM/F-12 stored PCLS was 51 ± 5% and 48 ± 6%, with and without addition of iron chelators, respectively. After storage in the chloride-rich *solution 1* and its chloride-poor analog *solution 2*, with and without iron chelators, the viability decreased to a lesser extent and was ~60% even after 14 days with a tendency for higher viability after storage in the chloride-rich *solution 1*. Storage in DMEM/F-12 led to continuously decreased viability with ongoing storage time to 8 ± 3% in the presence, and 4 ± 1% in the absence of iron chelators on *day 14*, which was significantly different to PCLS stored in *solution 1* ($P < 0.05$). After 28 days of cold storage of PCLS in DMEM/F-12 and subsequent rewarming, no resazurin reduction was detectable, indicating a complete loss of viability. Again, PCLS stored in *solution 1* (29 ± 7%) showed a tendency for higher viability compared with PCLS stored in *solution 2* (4 ± 2%). At all examined time points, no significant differences between solutions with and without addition of the chelators LK 614 and deferoxamine were observed (Fig. 2). Iron chelators showed beneficial effects for cold storage of vascular endothelial cells (28) and muscle tissue (25), which are also present in PCLS. In addition, iron chelators showed a tendency for higher viability in the current study and did not exert negative effects. Thus, the cold storage solutions were supplemented with the iron chelators LK 614 and deferoxamine in all subsequent experiments.

In addition to the assessment of the metabolic activity, cellular viability after 14 days of cold storage was evaluated by live/dead staining. After 14 days of cold storage, PCLS stored in DMEM/F-12 showed a strong reduction in viability and an increased number of dead cells (Fig. 3*B*). In contrast, PCLS stored in *solution 1* (Fig. 3*C*) and *solution 2* (Fig. 3*D*) exhibited a tissue viability comparable with unstored slices (Fig. 3*A*).

Mitochondrial Membrane Potential

Mitochondrial membrane potential of PCLS after 3 days of cold storage and rewarming was comparable with unstored PCLS for all preservation solutions (Fig. 4). After 7 days of cold storage in DMEM/F-12, the mitochondrial membrane potential was significantly lower (54 ± 12%; $P < 0.05$) than in nonstored controls (Fig. 5*A*) whereas that was not the case for PCLS stored in *solution 1* (83 ± 12%) or *solution 2* (74 ± 9%). After 14 days, however, there was also a significant difference ($P < 0.05$) of the mitochondrial membrane potential of PCLS stored in DMEM/F-12 (18 ± 5%; Fig. 5*B*) compared with PCLS stored in *solution 1* (95 ± 5%; Fig. 5*C*). Fourteen days of cold storage in *solution 2* resulted in 60 ± 5% mitochondrial membrane potential (Fig. 5*D*). After 28 days, the mitochondrial membrane potential of PCLS stored in *solution 1* was still 56 ± 5% of unstored control which was significantly higher than after cold storage in *solution 2* (9 ± 1%; $P < 0.05$) or DMEM/F-12 (1 ± 1%; Fig. 4).

Preservation of Specific Cell Populations

Single-cell suspension of unstored PCLS contained a total of $3.73 \pm 0.32 \times 10^6$ cells per 20 PCLS. After cold storage, cell numbers significantly decreased in DMEM/F-12 stored samples after 7 (1.46 ± 0.25 mio. cells; $P < 0.001$ vs. unstored) and 14 days (0.51 ± 0.16 mio. cells; $P < 0.001$ vs. unstored). PCLS cold-stored in *solution 2* contained a total of 2.39 ± 0.09 mio. cells after 7 days ($P < 0.01$ vs. unstored), and 1.89 ± 0.16 mio. after 14 days ($P < 0.001$ vs. unstored). In contrast, 2.83 ± 0.11 mio. cells (*day 7*) and 2.75 ± 0.24 mio. cells ($P < 0.05$ vs. unstored; *day 14*) were present after storage in *solution 1*. After 7 and 14 days, cell numbers were significantly higher in PCLS stored in *solution 1* than in DMEM/F-12 ($P < 0.05$). Furthermore, storage in *solution 2* preserved a higher number of cells than DMEM/F-12 after 14 days of cold storage (Fig. 6*A*; $P < 0.05$). Staining for inflammatory (CD45), epithelial (EpCAM), endothelial (CD31), or mesenchymal (CD90) cell types without storage revealed a proportion of 23.7 ± 1.9% $CD45^+$, 14.4 ± 0.9% $EpCAM^+$, 25.3 ± 3.3% $CD31^+$, and 14.7 ± 1.8% $CD90^+$ cells (Fig. 6*B*). Relation of $EpCAM^+$ (Fig. 6*C*) and $CD90^+$ cells (Fig. 6*D*) did not change during cold storage in any of the

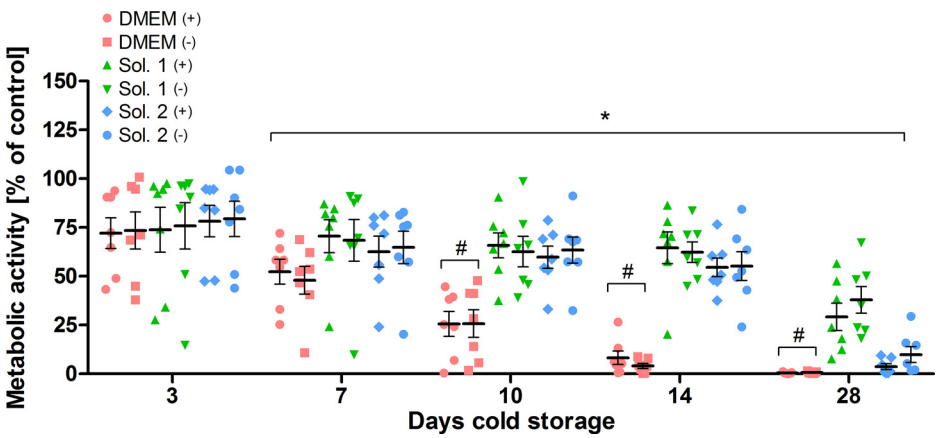

Figure 2. Alamar Blue conversion by precision-cut lung slices (PCLS) stored at 4°C in different cold storage solutions followed by rewarming. After preparation, PCLS were stored at 4°C for 3–28 days in DMEM/F-12, *solution 1* (chloride-rich) or *solution 2* (chloride-poor), either with (+) or without (−) addition of the chelators *N*-hydroxy-3,4-dimethoxy-*N*-methylbenzamide (LK 614, 0.02 mmol/L) and deferoxamine (0.1 mmol/L), followed by 3 h rewarming in DMEM/F-12 medium. Afterward, changes in viability were determined using an Alamar Blue assay and are shown as % of unstored PCLS. Data are presented as means ± SE ($n = 7$ PCLS from 7 different animals). Statistical significance: *$P < 0.05$ vs. control; #$P < 0.05$ vs. slices stored in *solution 1*.

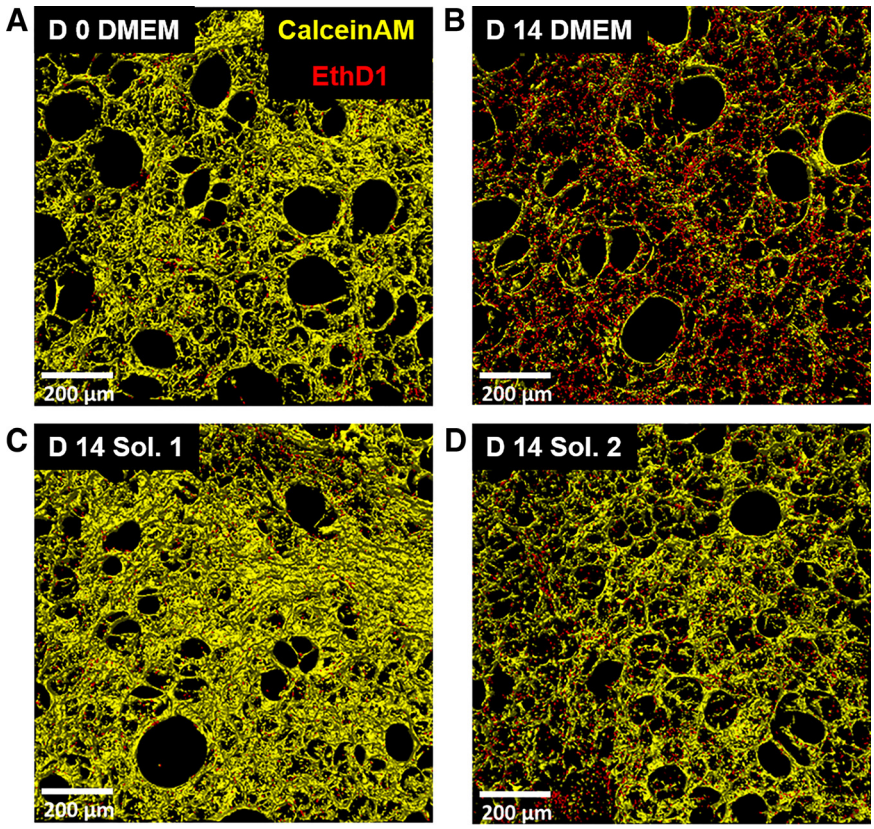

Figure 3. Representative images of live/dead staining in precision-cut lung slices (PCLS) stored at 4°C in different cold storage solutions followed by rewarming. After preparation, PCLS were either analyzed directly (*A*) or stored for 14 days at 4°C in DMEM/F-12 (*B*), *solution 1* (chloride-rich; *C*), or *solution 2* (chloride-poor; *D*), supplemented with the chelators *N*-hydroxy-3,4-dimethoxy-*N*-methylbenzamide (LK 614, 0.02 mmol/L) and deferoxamine (0.1 mmol/L), followed by 3 h rewarming in DMEM/F-12 medium. Afterward, PCLS were stained with calcein acetoxymethyl (calcein AM, yellow, indicating the cytosol of living cells) or ethidium homodimer-1 (EthD1, red, indicating nuclei of dead cells) in DMEM/F-12 and analyzed by confocal microscopy (×100 magnification, scale bar = 200 µm). Images were created by three-dimensional (3-D) surface rendering of Z-stacks and are representative examples of n = 3 PCLS from three different animals.

applied storage solutions. In contrast, proportion of $CD45^+$ cells significantly decreased after 7 days of cold storage in DMEM/F-12 (13.0 ± 1.9%; $P < 0.01$ vs. unstored), whereas storage in *solution 1* or *solution 2* led to a better preservation (18.1 ± 2.0% and 16.0 ± 1.9%, respectively, not significant). After 14 days of cold storage, $CD45^+$ cells further decreased to 11.3 ± 1.3% (*solution 1*) and 12.7 ± 1.1% (*solution 2*), whereas DMEM/F-12 stored PCLS did not contain enough viable cells for analysis (Fig. 6*E*). Proportion of $CD31^+$ cells was only significantly decreased after storage for 14 days in *solution 2* (13.9 ± 1.0%; Fig. 6*F*).

TNF-α Expression

Unstored PCLS expressed only low amounts of TNF-α after 24 h of incubation in DMEM/F-12 at 37°C (9.2 ± 2.1 pg/mL). In contrast, TNF-α expression was significantly ($P < 0.001$) increased after stimulation for 24 h with 100 ng/mL LPS (164.6 ± 27.0 pg/mL; Fig. 7*A*). No change in cytokine expression was observed after storage for up to 28 days in DMEM/F-12, *solution 1*, or *solution 2* without LPS stimulation. Significant TNF-α expression after LPS stimulation was observed in PCLS stored in DMEM/F-12 (65.6 ± 12.3 pg/mL) or

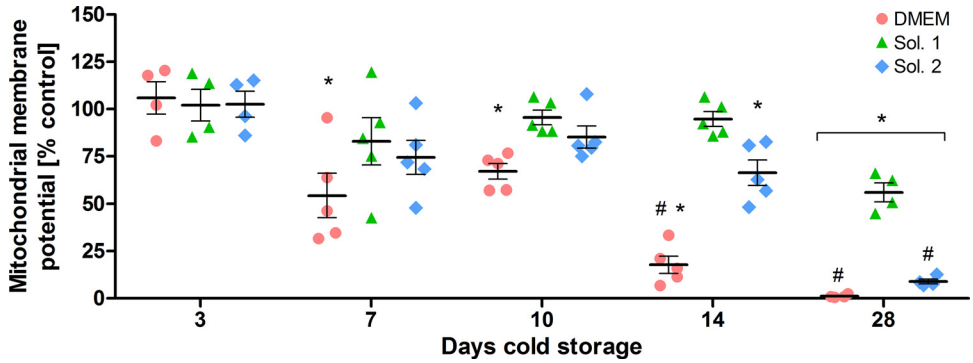

Figure 4. Mitochondrial membrane potential in precision-cut lung slices (PCLS) stored at 4°C in different cold storage solutions followed by rewarming. After preparation, PCLS were stored at 4°C for 3–28 days in DMEM/F-12, *solution 1* (chloride-rich), or *solution 2* (chloride-poor), supplemented with the iron chelators *N*-hydroxy-3,4-dimethoxy-*N*-methylbenzamide (LK 614, 0.02 mmol/L) and deferoxamine (0.1 mmol/L), followed by 3 h rewarming in DMEM/F-12 medium. Afterward, mitochondrial membrane potential was assessed using a tetramethylrhodamine methyl ester (TMRM) staining with subsequent confocal microscopy. Mean fluorescence intensity was calculated using ImageJ software and is shown as % of unstored PCLS. Data are presented as means ± SE ($n_{(D3,\ D28)}$ = 4; $n_{(D7,\ D10,\ D14)}$ = 5 PCLS from 4 or 5 different animals). Statistical significance: *$P < 0.05$ vs. unstored slices; #$P < 0.05$ vs. slices stored in *solution 1*.

AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00076.2021 • www.ajplung.org

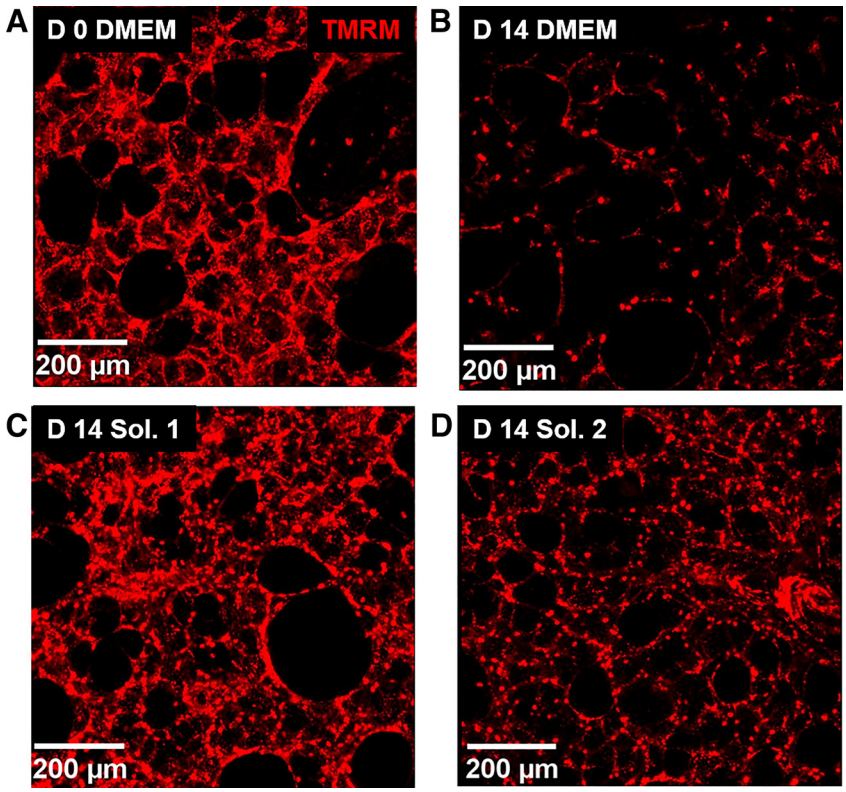

Figure 5. Representative images of tetramethylrhodamine methyl ester (TMRM)-stained precision-cut lung slices (PCLS) after 14 days of cold storage followed by rewarming. After preparation, PCLS were either analyzed directly (*A*) or stored for 14 days at 4°C in DMEM/F-12 (*B*), *solution 1* (*C*), or *solution 2* (*D*) followed by 3 h rewarming in DMEM/F-12 medium. Afterward, mitochondrial membrane potential was assessed by TMRM staining (red, indicating the presence of mitochondrial membrane potential) with subsequent confocal microscopy (×100 magnification, scale bar = 200 µm).

solution 2 (102.7 ± 32.7 pg/mL; Fig. 7*C*) after 3 days but decreased substantially after 7 days of cold storage. In contrast, a significant LPS-dependent TNF-α expression was observable in *solution 1*-stored slices for up to 10 days of storage (45.3 ± 13.6 pg/mL; Fig. 7*B*, $P < 0.05$ vs. unstimulated control).

Bronchoconstriction

As additional, functional parameter for the quality of PCLS after cold storage, the ability of airways in PCLS to constrict upon acetylcholine stimulus was analyzed (Fig. 8). A massive impairment of bronchoconstriction induced by cold storage in DMEM/F-12 was observed between *day 10* and *14*, with an almost complete loss of bronchoconstriction (4.7 ± 2.6%). In addition, bronchoconstriction was significantly impaired after cold storage of PCLS in *solution 2* (59.9 ± 9.4%; $P < 0.05$ vs. unstored) whereas storage in *solution 1* resulted in bronchoconstriction very similar to control values. The constriction after ACh stimulus further decreased after storage in DMEM/F-12 (5.9 ± 2.1%) and in *solution 2* (13.0 ± 3.2%) on *day 28* but was still 74.3 ± 11.7% in *solution 1*-stored PCLS. As a secondary finding during bronchoconstriction experiments, ciliary beating was observed in all slices directly after preparation and after 3 days of cold storage but substantially decreased after 7 days of cold storage in DMEM/F-12 until no beating was observed after 14 days of storage. In contrast, 92% of the airways of PCLS stored in *solution 1* showed ciliary beating after 28 days of cold storage. Ciliary beating in airways of PCLS stored in *solution 2* was also preserved in part of the slices after 28 days of storage (58%).

DISCUSSION

To provide optimal tissue protection during PCLS cold storage, we investigated the protecting properties of standard culture medium DMEM/F-12 as well as of a potassium and chloride-rich tissue preservation solution (*solution 1*, TiProtec) and a chloride-poor, lactobionate-rich version thereof (*solution 2*; detailed composition of the solutions can be found in Table 1). *Solution 1* has been optimized for cold storage of vascular grafts (28), but subsequently also proved to be beneficial for cold storage of muscle tissue (25) and human hepatocytes (29). Its chloride-poor derivative *solution 2* yielded superior protection for rat hepatocytes during prolonged cold storage (24). In addition, use of chloride-rich preservation solutions was found to be beneficial for the preservation of human epithelial cell lines and mice lung tissue samples after cold storage for 3 days (30).

Reducing the preservation temperature is essential to suppress metabolic rate, sustain energy supply, and enhance defense mechanisms, especially when oxygen supply is limited (31). As indicated by the Alamar Blue assay, metabolic activity is highly reduced in all storage solutions during cold storage compared with unstored control PCLS (between 4.0 ± 0.8% and 6.0 ± 1.3%). Under ischemic/hypoxic conditions, oxidative phosphorylation ceases and adenosine triphosphate (ATP) is consumed rapidly during cold storage, resulting in impaired cellular homeostasis and cell function finally causing cellular death (32). Additional cold storage-dependent injury may arise due to the generation of reactive oxygen species (ROS) or increased cytokine production (33, 34). An important underlying mechanism of cold storage-

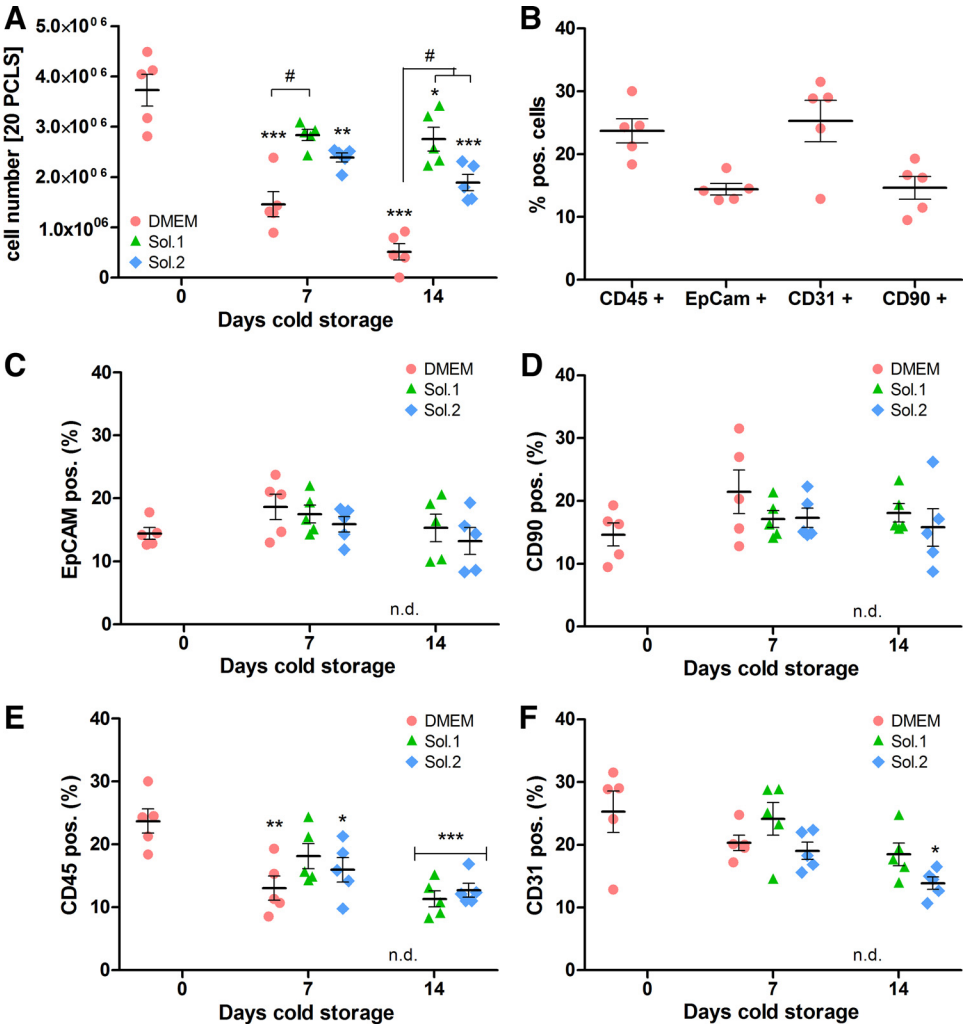

Figure 6. Proportion of cell types present in precision-cut lung slices (PCLS) stored at 4°C in different cold storage solutions followed by rewarming. After preparation, 20 PCLS were either analyzed directly or stored for 14 days at 4°C in DMEM/F-12, *solution 1* (chloride-rich), or *solution 2* (chloride-poor), supplemented with the chelators *N*-hydroxy-3,4-dimethoxy-*N*-methylbenzamide (LK 614, 0.02 mmol/L) and deferoxamine (0.1 mmol/L), followed by 3 h rewarming in DMEM/F-12 medium. Afterward, single-cell suspensions were prepared, cells were counted (*A*) and proportions of $CD45^+$, $EpCam^+$, $CD31^+$, or $CD90^+$ cells, either directly after preparation (*B*) or after 7 and 14 days of cold storage (*C*–*F*), were analyzed. Data are presented as means ± SE (n = 5 samples from 5 different animals). Statistical significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. unstored PCLS, $\#P < 0.05$ vs. DMEM stored PCLS; n.d., not detectable due to low cell number.

dependent ROS formation is an increase in the cellular chelatable iron pool contributing to cold-induced apoptosis (35).

To counteract cold-induced iron-dependent injury, iron chelators were added to the preservation solutions. The small lipophilic iron chelator LK 614 is used to enter cells in the early phase of cold storage and chelate iron before ROS are generated. The large hexadentate chelator deferoxamine has been found to prevent endothelial dysfunction in early stages of cold storage (28). Deferoxamine is a known reducer of iron-dependent ROS formation (36, 37) that acts via complex formation with Fe^{3+} iron. In this study, we were not able to detect significant improvement of viability by the addition of deferoxamine and LK 614 to preservation solutions. However, addition of both chelators led to a nonsignificant improvement of mitochondrial membrane potential of DMEM/F-12 after 10 days (67 ± 4% vs. 50 ± 5%) and *solution 2* after 14 days (66 ± 7% vs. 52 ± 3%; data not shown for chelator-free solutions in Fig. 4) of cold storage. It has already been shown that deferoxamine and LK 614 can prevent mitochondrial alterations during cold storage of rat proximal tubular cells (38), A549 lung epithelial cells (26), and hepatocytes (24, 29). However, previous studies suggested a higher sensitivity of cultured cells toward cold preservation iron-dependent injury compared with more complex tissues or to cells in suspension (24). This might explain why no significant changes occurred during the first 3 days of storage (Fig. 2) and why the effect of iron chelators was small in the present study. Furthermore, A549 cells are adenocarcinomic alveolar cells and elevated ROS production is observed in almost all cancers (39), suggesting also a stronger effect of ROS and thus of antioxidative self-defense strategies.

Warm storage of PCLS at 37°C and 5% CO_2 without medium change resulted in a significant loss of viability from *day 7* to *day 14*, suggesting depletion of energy and loss of buffer capacity (40). The live span of PCLS can be improved for up to 14 days by the introduction of daily medium exchange (12). However, for transport and shipment, maintenance of 37°C and gassing with 5% CO_2 is challenging. Therefore, it was reasonable to evaluate the viability after long-term storage at 4°C in cell culture medium. Cold storage in DMEM/F-12 culture medium and rewarming led to a continuous loss of viability resulting in 30 ± 10% after 10 days, which is substantially lower than in PCLS stored permanently at 37°C (58 ± 14%). The process of cooldown to 4°C, maintenance of this temperature during storage, and the subsequent rewarming in culture medium has been found to induce blebbing of the plasma membrane, cell detachment, and DNA fragmentation finally resulting in apoptosis (41).

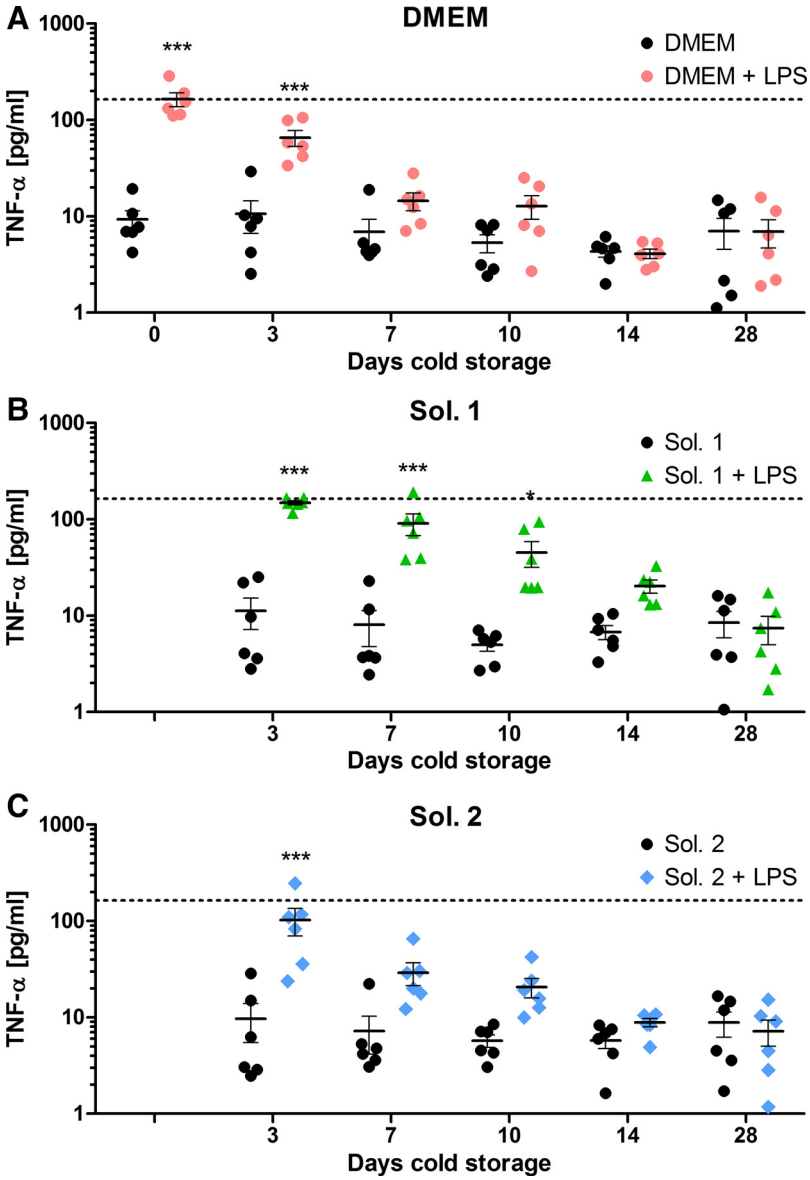

Figure 7. Release of tumor necrosis factor-α (TNF-α) from precision-cut lung slices (PCLS) stored at 4°C in different storage solutions followed by rewarming with or without lipopolysaccharide (LPS) stimulation for 24 h. After preparation, PCLS were either exposed directly to DMEM/F-12 with, or without 100 ng/mL LPS or stored for 14 days at 4°C in DMEM/F-12 (*A*), *solution 1* (chloride-rich; *B*), or *solution 2* (chloride-poor; *C*), supplemented with the iron chelators *N*-hydroxy-3,4-dimethoxy-*N*-methylbenzamide (LK 614, 0.02 mmol/L) and deferoxamine (0.1 mmol/L), followed by 3 h rewarming in DMEM/F-12 medium. Afterward, expression of TNF-α in the supernatant of unstimulated and LPS exposed PCLS was detected by ELISA. Cytokine expression is shown in pg/mL supernatant, dashed line (---) shows the TNF-α expression without cold storage after LPS stimulation. Data are presented as means ± SE, $n = 6$ PCLS from 6 different animals. Statistical significance: $^{*}P < 0.05$, $^{***}P < 0.001$ vs. PCLS incubated with medium without LPS.

During cold storage, function of ion pumps like the Na^+/K^+-ATPase is reduced, which has been suggested to impede maintenance of ion equilibrium leading to cell swelling and necrosis (42). However, in the presence of iron chelators, cold storage in chloride-rich preservation solutions, with chloride concentrations comparable with cell culture medium, proved to be superior to cold storage in chloride-poor organ preservation solutions for cell types such as rat proximal tubules and porcine aortic endothelial cells (28, 43).

To provide an optimal environment for cold storage of tissues, improved preservation solutions were applied containing additional protective compounds. In our experiments, both solutions significantly decreased cell injury compared with DMEM/F-12-stored PCLS after long-term hypothermic storage (Fig. 2). Evaluation of live/dead staining, which is frequently used for analysis of PCLS viability (12, 44), revealed viable tissue after 14 days of cold storage in *solution 1* and *solution 2*, that was comparable with unstored PCLS. In contrast, PCLS cold stored in DMEM/F-12 contained only a few viable cells but a high number of EthD1-stained nuclei, indicating substantial cell death which is in line with the metabolic activity (cf. Figs. 2 and 3).

Mitochondria are thought to play a crucial role in apoptosis and necrosis after cold storage and lose membrane potential during rewarming. Furthermore, swelling of mitochondria and loss of inner mitochondrial membrane was observed after 24 h cold storage of proximal tubular cells (29, 38). Cold storage is known to induce ROS production (45–47) associated with mitochondrial damage (47, 48). Therefore, we analyzed the mitochondrial membrane potential after cold storage and rewarming. After 3 days of storage, no change in membrane potential was observed, thus the process of cooldown and rewarming seems to have no direct negative impact on mitochondria during early phase of cold storage (Fig. 4). *Solutions 1* and *2* both preserved mitochondria significantly better than standard culture medium after 14 days, which underlines the need for the earlier discussed supplements during cold storage of PCLS.

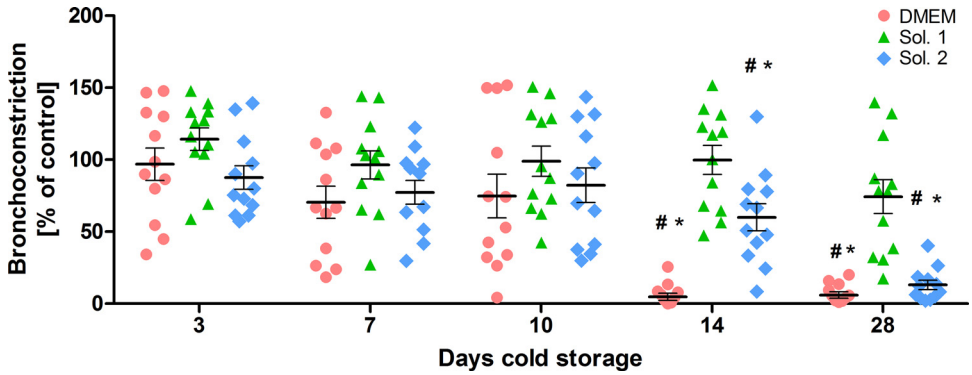

Figure 8. Airway constriction in precision-cut lung slices (PCLS) stored at 4°C in different cold storage solutions followed by rewarming. After preparation, PCLS were stored for up to 28 days at 4°C in DMEM/F-12, *solution 1* (chloride-rich), or *solution 2* (chloride-poor), supplemented with the chelators *N*-hydroxy-3,4-dimethoxy-*N*-methylbenzamide (LK 614, 0.02 mmol/L) and deferoxamine (0.1 mmol/L) followed by 3 h rewarming in DMEM/F-12 medium. Afterward, airway constriction upon acetylcholine (ACh) stimulus (0.5 µmol/L) and 2-min incubation was examined using an inverted microscope. Cross-sectional airway-area was calculated before as well as 2 min after addition of ACh. Thereof, percentage of bronchoconstriction was calculated and expressed as relation to constriction of unstored PCLS. Data are presented as means ± SE ($n = 12$ airways from at least 4 different animals). Statistical significance: $*P < 0.05$ vs. unstored slices. $\#P < 0.05$ vs. *solution 1*-stored PCLS.

As the aforementioned parameters were used to study the overall tissue viability without taking the distribution of specific cell types into account, we analyzed whether cold storage in the modified solutions or DMEM/F-12 has effects on the cell composition of PCLS. Single-cell suspensions of PCLS were prepared and stained for $CD45^+$ immune cells, $EpCAM^+$ epithelial cells, $CD31^+$ endothelial cells, and $CD90^+$ mesenchymal cells to evaluate cell populations that are of interest for PCLS research (49–51). Cell numbers of PCLS stored in DMEM/F-12 were significantly lower than after storage in *solution 1* at *day 7* and after storage in both modified solutions at *day 14*, which is in line with the tissue viability analyzed by Alamar Blue conversion (Fig. 6*A*). This is of special importance because single-cell suspensions from PCLS are used for the investigation of endpoints like immune cell activation or live/dead staining (13, 52).

The observed proportion of cell populations is in line with observations from other groups, where $CD45^+$ cells are represented in high numbers, whereas only a small proportion of cells is EpCAM-positive (53, 54). However, the proportion of cell types observed in single-cell suspensions is highly dependent on the protocol and enzymes used for digestion, which may affect cell dissociation efficiency but also the presence or absence of surface markers (53, 55). Evaluation of immunofluorescence staining data of the single-cell suspensions revealed that the proportions of epithelial ($EpCAM^+$) and mesenchymal ($CD90^+$) cells are not affected by cold storage in the different storage solutions (Fig. 6). In addition, proportion of endothelial cells ($CD31^+$) remained unchanged during storage for 7 days and was only significantly decreased after 14 days of storage in *solution 2*, while no such effect was observed after storage in *solution 1*. Strongest effects of cold storage were seen analyzing the population of $CD45^+$ immune cells (Fig. 6*E*). Thereby, proportion of immunological cells was significantly decreased after cold storage for 7 days in DMEM/F-12, while a less strong decrease was observed after storage in the modified storage solutions. The proportion significantly decreased after storage for 14 days in *solution 1* and *solution 2* and revealed that inflammatory cells like macrophages are more susceptible toward cold storage injury than epithelial, endothelial, or mesenchymal cell populations. The use of modified storage solutions can not only preserve a higher number of cells after cold storage in general but can also protect sensitive immune cells better than standard DMEM/F-12 culture medium. Furthermore, maintenance of the cellular composition enables the use of cold-stored PCLS for metabolomic, proteomic, or transcriptomic approaches (51, 56).

As the population of inflammatory cells decreased after cold storage in all solutions, we analyzed the basal inflammatory activation level and whether PCLS responded to an inflammatory stimulus. Analysis of inflammatory activation is a major advantage of PCLS to study the cellular response toward viral infections or chemical exposures. Thereby, the amount of inflammatory cells in PCLS is comparable with the amount of resident inflammatory cells in vivo which enables a realistic evaluation of inflammatory response (1). Basal TNF-α expression, as marker for inflammatory tissue injury (57), was not increased after cold storage in any of the storage solutions, indicating a minor effect of inflammatory activation (cf. Fig. 7*A*, 0 days vs. other tested conditions). After cold storage for 10 days and subsequent LPS stimulation to induce an inflammatory response (58), PCLS cold-stored in *solution 1* released significantly higher concentrations of TNF-α compared with slices without LPS stimulation. In contrast, PCLS stored in DMEM/F-12 or *solution 2* showed no inflammatory response when stored for longer than 3 days (Fig. 7). Therefore, PCLS cold-stored in *solution 1* respond substantially longer to an inflammatory stimulus than PCLS stored in the other solutions. Compared with overall viability, the inflammatory response after cold storage and rewarming decreased faster in all storage solutions but is well comparable with the proportion of inflammatory cells (compare Fig. 2 vs. Fig. 6 vs. Fig. 7). Inflammatory response of macrophages seems to be more sensitive toward cold storage than overall tissue viability and is in line with data of long-term PCLS warm storage (12). Our data indicate that a chloride-rich environment is essential for long-term cold preservation of inflammatory cells in PCLS and that

chloride substitution by lactobionate is not beneficial to preserve functional inflammatory response.

Besides evaluation of viability and inflammatory activity, PCLS are frequently used for the detection of airway constriction. Concurrently, constriction of airways requires intact tissue architecture and cooperation of different cellular structures which allows a general evaluation of PCLS functionality (6, 12, 59). Our results show well preserved airway response for up to 10 days in all storage solutions (Fig. 8). Keeping in mind that the lung tissue got stressed by agarose filling, slicing, cooldown, cold storage, and rewarming, these results remarkably underline the robustness of the PCLS model. Strong differences were observed after 14 days of cold storage. Although the overall cellular viability, detected via Alamar Blue assay, decreased significantly already after 10 days of storage in DMEM/F-12, bronchoconstriction was fully preserved (cf. Fig. 2 and Fig. 8). This suggests that the airways maintained the ability to constrict, even if overall viability was already decreased. The optimized storage solutions preserved ciliary beating after 28 days of cold storage and rewarming, which is a major improvement toward DMEM/F-12-stored slices that lost ciliary beating completely after 14 days. It is important to note, that only the presence or absence of ciliary beating was visually assessed in the current study but not the speed or frequency of ciliary beating. Future studies should evaluate the beating frequency after cold storage using a high-frame rate camera (8). The aforementioned preservation solutions contain various compounds to address different mechanisms of injury: addition of glycine and alanine to the solutions limits the increase of cytosolic sodium via prevention of nonspecific leak formation, which was found to be protective against hypoxic cell injury that might occur in submersed PCLS (60, 61). However, hypoxic injury is unlikely to occur during PCLS cold storage as the tissue sections are thin (~250 μm) and solubility of oxygen in the storage solutions is increased at lower temperatures. In addition, oxygen consumption during cold storage is decreased and it has been found that low levels of oxygen are beneficial for the preservation of mitochondrial function in cold storage of whole lung (62). Hypoxic injury might play a stronger role for thicker PCLS, that are often prepared from human tissue (>500 μm), and for cold storage of tissue cores before slicing. Tryptophan and α-ketoglutarate are major ingredients of the histidine-tryptophan-ketoglutarate solution used in organ transplantation to stabilize cellular membranes and to serve as substrate for ATP production, which is limited during cold storage (63). In addition, α-ketoglutarate has been found to reduce the generation of hydrogen peroxide, thus it can help to provide protection against reactive oxygen species (64). Aspartate helps to replenish the tricarboxylic acid cycle securing energy supply. Furthermore, to maintain the optimal buffering conditions, the naturally occurring histidine derivate *N*-acetylhistidine was added, which is less cytotoxic than high concentrations of histidine or of phosphate (28, 65). Control of osmotic cell swelling during preservation is essential to maintain cellular viability, which can be achieved by the addition of impermeable osmolytes like sucrose (66), an acidic pH is known to offer protection against hypoxic injury (67), and a low calcium concentration can provide protection against diverse types of injury (68, 69).

Although *solution 1* contains a high physiological extracellular concentration of chloride, the major part of chloride was substituted in *solution 2* by the large organic anion lactobionate (Table 1). *Solution 1* provided significantly better preservation after 14 days of cold storage compared with *solution 2* regarding the functional parameters mitochondrial membrane potential and bronchoconstriction and preserved inflammatory response for longer time. Although 14 days substantially extend shipment durations it is obvious that the PCLS have functional limitations before the cells finally die. Chloride efflux has been found to occur during early stages of cold storage, which can be diminished by high extracellular chloride concentrations used in *solution 1* (70). Lactobionate has been found to prevent tissue edema formation during cold storage (71) but had minor impact on overall tissue viability than using chloride as main anion in our experiments. Therefore, choosing chloride rather than lactobionate as main anion in the improved base solution provides substantial improvement during cold storage of PCLS.

Conclusions

Taken together, the optimized potassium chloride-rich preservation *solution 1* provided superior tissue protection to its chloride-poor analog *solution 2* and standard culture medium DMEM/F-12 for long-term cold storage and shipment of PCLS.

ACKNOWLEDGMENTS

The authors thank Dr. M. Lehmann and Q. Hu for advice in preparation of single-cell suspensions and FACS analysis. Furthermore, the authors thank L. Seck for excellent assistance in PCLS preparation and measurement of bronchoconstriction.

Present address for T. Wille: Bundeswehr Medical Academy, Ingolstädter Straße 240, 80939 Munich, Germany.

GRANTS

This work was supported from the German Research Foundation (GRK 2338, Targets in Toxicology); project P02 (to T.W. and F.W.); J.T. received a PhD stipend in the GRK 2338.

DISCLOSURES

U.R. has been a consultant of Dr. Franz Köhler Chemie and is listed as one of the inventors in the patents the company holds on the preservation solutions Custodiol-N, TiProtec, and Custodiol-MP. U.R. received some funding for scientific projects by the company, but this is unrelated to the current study. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

J.T., U.R., and T.W. conceived and designed research; J.T. performed experiments; J.T. analyzed data; J.T., U.R., and T.W. interpreted results of experiments; J.T. prepared figures; J.T. drafted manuscript; F.E., F.W., H.T., U.R., and T.W. edited and revised manuscript; J.T., F.E., F.W., H.T., U.R., and T.W. approved final version of manuscript.

REFERENCES

1. **Liu G**, **Betts C**, **Cunoosamy DM**, **Åberg PM**, **Hornberg JJ**, **Sivars KB**, **Cohen TS.** Use of precision cut lung slices as a translational

model for the study of lung biology. *Respir Res* 20: 162, 2019. doi:10.1186/s12931-019-1131-x.

2. **Parrish AR**, **Gandolfi A**, **Brendel K.** Precision-cut tissue slices: applications in pharmacology and toxicology. *Life Sci* 57: 1887–1901, 1995. doi:10.1016/0024-3205(95)02176-J.
3. **Behrsing HP**, **Furniss MJ**, **Davis M**, **Tomaszewski JE**, **Parchment RE.** In vitro exposure of precision-cut lung slices to 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole lysylamide dihydrochloride (NSC 710305, Phortress) increases inflammatory cytokine content and tissue damage. *Toxicol Sci* 131: 470–479, 2013. doi:10.1093/toxsci/kfs319.
4. **Hirn S**, **Haberl N**, **Loza K**, **Epple M**, **Kreyling WG**, **Rothen-Rutishauser B**, **Rehberg M**, **Krombach F.** Proinflammatory and cytotoxic response to nanoparticles in precision-cut lung slices. *Beilstein J Nanotechnol* 5: 2440–2449, 2014. doi:10.3762/bjnano.5.253.
5. **Henjakovic M**, **Sewald K**, **Switalla S**, **Kaiser D**, **Müller M**, **Veres TZ**, **Martin C**, **Uhlig S**, **Krug N**, **Braun A.** Ex vivo testing of immune responses in precision-cut lung slices. *Toxicol Appl Pharmacol* 231: 68–76, 2008. doi:10.1016/j.taap.2008.04.003.
6. **Herbert J**, **Thiermann H**, **Worek F**, **Wille T.** Precision cut lung slices as test system for candidate therapeutics in organophosphate poisoning. *Toxicology* 389: 94–100, 2017. doi:10.1016/j.tox.2017.07.011.
7. **Herbert J**, **Thiermann H**, **Worek F**, **Wille T.** COPD and asthma therapeutics for supportive treatment in organophosphate poisoning. *Clin Toxicol (Phila)* 57: 644–651, 2019. doi:10.1080/15563650.2018.1540785.
8. **Wohlsen A**, **Martin C**, **Vollmer E**, **Branscheid D**, **Magnussen H**, **Becker WM**, **Lepp U**, **Uhlig S.** The early allergic response in small airways of human precision-cut lung slices. *Eur Respir J* 21: 1024–1032, 2003. doi:10.1183/09031936.03.00027502.
9. **Fryer AD**, **Lein PJ**, **Howard AS**, **Yost BL**, **Beckles RA**, **Jett DA.** Mechanisms of organophosphate insecticide-induced airway hyperreactivity. *Am J Physiol Lung Cell Mol Physiol* 286: L963–L969, 2004. doi:10.1152/ajplung.00343.2003.
10. **Liberati TA**, **Randle MR**, **Toth LA.** In vitro lung slices: a powerful approach for assessment of lung pathophysiology. *Expert Rev Mol Diagn* 10: 501–508, 2010. doi:10.1586/erm.10.21.
11. **Russell WMS**, **Burch RL.** *The Principles of Humane Experimental Technique*. London: Methuen, 1959.
12. **Neuhaus V**, **Schaudien D**, **Golovina T**, **Temann U-A**, **Thompson C**, **Lippmann T**, **Bersch C**, **Pfennig O**, **Jonigk D**, **Braubach P**, **Fieguth H-G**, **Warnecke G**, **Yusibov V**, **Sewald K**, **Braun A.** Assessment of long-term cultivated human precision-cut lung slices as an ex vivo system for evaluation of chronic cytotoxicity and functionality. *J Occup Med Toxicol* 12: 13, 2017. doi:10.1186/s12995-017-0158-5.
13. **Li G**, **Cohen JA**, **Martines C**, **Ram-Mohan S**, **Brain JD**, **Krishnan R**, **Ai X**, **Bai Y.** Preserving airway smooth muscle contraction in precision-cut lung slices. *Sci Rep* 10: 6480, 2020. doi:10.1038/s41598-020-63225-y.
14. **Bai Y**, **Krishnamoorthy N**, **Patel KR**, **Rosas I**, **Sanderson MJ**, **Ai X.** Cryopreserved human precision-cut lung slices as a bioassay for live tissue banking. A viability study of bronchodilation with bitter-taste receptor agonists. *Am J Respir Cell Mol Biol* 54: 656–663, 2016. doi:10.1165/rcmb.2015-0290MA.
15. **Baatz JE**, **Newton DA**, **Riemer EC**, **Denlinger CE**, **Jones EE**, **Drake RR**, **Spyropoulos DD.** Cryopreservation of viable human lung tissue for versatile post-thaw analyses and culture. *In vivo* 28: 411–423, 2014.
16. **Rosner SR**, **Ram-Mohan S**, **Paez-Cortez JR**, **Lavoie TL**, **Dowell ML**, **Yuan L**, **Ai X**, **Fine A**, **Aird WC**, **Solway J**, **Fredberg JJ**, **Krishnan R.** Airway contractility in the precision-cut lung slice after cryopreservation. *Am J Respir Cell Mol Biol* 50: 876–881, 2014. doi:10.1165/rcmb.2013-0166MA.
17. **Bakhach J.** The cryopreservation of composite tissues: principles and recent advancement on cryopreservation of different type of tissues. *Organogenesis* 5: 119–126, 2009. doi:10.4161/org.5.3.9583.
18. **Elliott GD**, **Wang S**, **Fuller BJ.** Cryoprotectants: a review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. *Cryobiology* 76: 74–91, 2017. doi:10.1016/j.cryobiol.2017.04.004.
19. **Pegg DE.** Principles of cryopreservation. *Methods Mol Biol* 1257: 3–19, 2015. doi:10.1007/978-1-4939-2193-5_1.
20. **Watson CY**, **Damiani F**, **Ram-Mohan S**, **Rodrigues S**, **de Moura Queiroz P**, **Donaghey TC**, **Rosenblum Lichtenstein JH**, **Brain JD**, **Krishnan R**, **Molina RM.** Screening for chemical toxicity using cryopreserved precision cut lung slices. *Toxicol Sci* 150: 225–233, 2016. doi:10.1093/toxsci/kfv320.
21. **Fisher RL**, **Hasal SJ**, **Sanuik JT**, **Hasal KS**, **Gandolfi AJ**, **Brendel K.** Cold- and cryopreservation of dog liver and kidney slices. *Cryobiology* 33: 163–171, 1996. doi:10.1006/cryo.1996.0016.
22. **Hart N'**, **van der Plaats A**, **Faber A**, **Leuvenink HGD**, **Olinga P**, **Wiersema-Buist J**, **Verkerke GJ**, **Rakhorst G**, **Ploeg RJ.** Oxygenation during hypothermic rat liver preservation: an in vitro slice study to demonstrate beneficial or toxic oxygenation effects. *Liver Transpl* 11: 1403–1411, 2005. doi:10.1002/lt.20510.
23. **Evdokimova E**, **Taper H**, **Buc Calderon P.** Effect of cold hypoxic storage and reoxygenation at 37°C of cultured precision-cut rat liver slices on paracetamol metabolism. *Arch Toxicol* 75: 452–458, 2001. doi:10.1007/s002040100265.
24. **Pless-Petig G**, **Singer BB**, **Rauen U.** Cold storage of rat hepatocyte suspensions for one week in a customized cold storage solution—preservation of cell attachment and metabolism. *PLoS One* 7: e40444, 2012. doi:10.1371/journal.pone.0040444.
25. **Wille T**, **Gonder S**, **Thiermann H**, **Seeger T**, **Rauen U**, **Worek F.** Evaluation of functional and structural alterations in muscle tissue after short-term cold storage in a new tissue preservation solution. *Cells Tissues Organs* 194: 501–509, 2011. doi:10.1159/000324148.
26. **Pizanis N**, **Gillner S**, **Kamler M**, **de Groot H**, **Jakob H**, **Rauen U.** Cold-induced injury to lung epithelial cells can be inhibited by iron chelators—implications for lung preservation. *Eur J Cardiothorac Surg* 40: 948–955, 2011. doi:10.1016/j.ejcts.2011.01.052.
27. **Hansen NUB**, **Karsdal MA**, **Brockbank S**, **Cruwys S**, **Rønnow S**, **Leeming DJ.** Tissue turnover of collagen type I, III and elastin is elevated in the PCLS model of IPF and can be restored back to vehicle levels using a phosphodiesterase inhibitor. *Respir Res* 17: 76, 2016. doi:10.1186/s12931-016-0394-8.
28. **Wille T**, **de Groot H**, **Rauen U.** Improvement of the cold storage of blood vessels with a vascular preservation solution. Study in porcine aortic segments. *J Vasc Surg* 47: 422–431, 2008. doi:10.1016/j.jvs.2007.09.048.
29. **Pless G**, **Sauer IM**, **Rauen U.** Improvement of the cold storage of isolated human hepatocytes. *Cell Transplant* 21: 23–37, 2012. doi:10.3727/096368911X580509.
30. **Suzuki T**, **Ota C**, **Fujino N**, **Tando Y**, **Suzuki S**, **Yamada M**, **Kondo T**, **Okada Y**, **Kubo H.** Improving the viability of tissue-resident stem cells using an organ-preservation solution. *FEBS Open Bio* 9: 2093–2104, 2019. doi:10.1002/2211-5463.12748.
31. **Guibert EE**, **Petrenko AY**, **Balaban CL**, **Somov AY**, **Rodriguez JV**, **Fuller BJ.** Organ preservation: current concepts and new strategies for the next decade. *Transfus Med Hemother* 38: 125–142, 2011. doi:10.1159/000327033.
32. **Blankensteijn JD**, **Terpstra OT.** Liver preservation: the past and the future. *Hepatology* 13: 1235–1250, 1991. doi:10.1002/hep.1840130633.
33. **Salahudeen AK**, **Huang H**, **Patel P**, **Jenkins JK.** Mechanism and prevention of cold storage-induced human renal tubular cell injury. *Transplantation* 70: 1424–1431, 2000. doi:10.1097/00007890-200011270-00005.
34. **Laubach VE**, **Sharma AK.** Mechanisms of lung ischemia-reperfusion injury. *Curr Opin Organ Transplant* 21: 246–252, 2016. doi:10.1097/MOT.0000000000000304.
35. **Rauen U**, **Petrat F**, **Li T**, **de Groot H.** Hypothermia injury/cold-induced apoptosis—evidence of an increase in chelatable iron causing oxidative injury in spite of low O_2-/H_2O_2 formation. *FASEB J* 14: 1953–1964, 2000. doi:10.1096/fj.00-0071com.
36. **Sharma BK**, **Bacon BR**, **Britton RS**, **Park CH**, **Magiera CJ**, **O'Neill R**, **Dalton N**, **Smanik P**, **Speroff T.** Prevention of hepatocyte injury and lipid peroxidation by iron chelators and alpha-tocopherol in isolated iron-loaded rat hepatocytes. *Hepatology* 12: 31–39, 1990. doi:10.1002/hep.1840120107.
37. **Octave J-N**, **Schneider Y-J**, **Crichton RR**, **Trouet A.** Iron mobilization from cultured hepatocytes: effect of desferrioxamine B. *Biochem Pharmacol* 32: 3413–3418, 1983. doi:10.1016/0006-2952(83)90370-2.
38. **Bartels-Stringer M**, **Kramers C**, **Wetzels JF**, **Russel FG**, **de Groot H**, **Rauen U.** Hypothermia causes a marked injury to rat proximal tubular cells that is aggravated by all currently used preservation

solutions. *Cryobiology* 47: 82–91, 2003. doi:10.1016/S0011-2240(03)00071-3.
39. **Liou G-Y**, **Storz P.** Reactive oxygen species in cancer. *Free Radic Res* 44: 479–496, 2010. doi:10.3109/10715761003667554.
40. **Krampe B**, **Al-Rubeai M.** Cell death in mammalian cell culture: molecular mechanisms and cell line engineering strategies. *Cytotechnology* 62: 175–188, 2010. doi:10.1007/s10616-010-9274-0.
41. **Rauen U**, **Polzar B**, **Stephan H**, **Mannherz HG**, **de Groot H.** Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species. *FASEB J* 13: 155–168, 1999. doi:10.1096/fasebj.13.1.155.
42. **Belzer FO**, **Southard JH.** Principles of solid-organ preservation by cold storage. *Transplantation* 45: 673–676, 1988. doi:10.1097/00007890-198804000-00001.
43. **Bienholz A**, **Walter B**, **Pless-Petig G**, **Guberina H**, **Kribben A**, **Witzke O**, **Rauen U.** Characterization of injury in isolated rat proximal tubules during cold incubation and rewarming. *PLoS One* 12: e0180553, 2017. doi:10.1371/journal.pone.0180553.
44. **Switalla S**, **Lauenstein L**, **Prenzler F**, **Knothe S**, **Förster C**, **Fieguth H-G**, **Pfennig O**, **Schaumann F**, **Martin C**, **Guzman CA**, **Ebensen T**, **Müller M**, **Hohlfeld JM**, **Krug N**, **Braun A**, **Sewald K.** Natural innate cytokine response to immunomodulators and adjuvants in human precision-cut lung slices. *Toxicol Appl Pharmacol* 246: 107–115, 2010. doi:10.1016/j.taap.2010.04.010.
45. **Awad EM**, **Khan SY**, **Sokolikova B**, **Brunner PM**, **Olcaydu D**, **Wojta J**, **Breuss JM**, **Uhrin P.** Cold induces reactive oxygen species production and activation of the NF-kappa B response in endothelial cells and inflammation in vivo. *J Thromb Haemost* 11: 1716–1726, 2013. doi:10.1111/jth.12357.
46. **Salehi P**, **Walker J**, **Madsen K**, **Churchill TA.** Control of oxidative stress in small bowel: relevance to organ preservation. *Surgery* 139: 317–323, 2006. doi:10.1016/j.surg.2005.08.003.
47. **Cruthirds DL**, **Novak L**, **Akhi KM**, **Sanders PW**, **Thompson JA**, **MacMillan-Crow LA.** Mitochondrial targets of oxidative stress during renal ischemia/reperfusion. *Arch Biochem Biophys* 412: 27–33, 2003. doi:10.1016/S0003-9861(03)00039-0.
48. **Saba H**, **Munusamy S**, **MacMillan-Crow LA.** Cold preservation mediated renal injury: involvement of mitochondrial oxidative stress. *Ren Fail* 30: 125–133, 2008. doi:10.1080/08860220701813327.
49. **Akram KM**, **Yates LL**, **Mongey R**, **Rothery S**, **Gaboriau DCA**, **Sanderson J**, **Hind M**, **Griffiths M**, **Dean CH.** Live imaging of alveologenesis in precision-cut lung slices reveals dynamic epithelial cell behaviour. *Nat Commun* 10: 1178, 2019. doi:10.1038/s41467-019-09067-3.
50. **Alsafadi HN**, **Uhl FE**, **Pineda RH**, **Bailey KE**, **Rojas M**, **Wagner DE**, **Königshoff M.** Applications and approaches for three-dimensional precision-cut lung slices. Disease modeling and drug discovery. *Am J Respir Cell Mol Biol* 62: 681–691, 2020. doi:10.1165/rcmb.2019-0276TR.
51. **Khan MM**, **Poeckel D**, **Halavatyi A**, **Zukowska-Kasprzyk J**, **Stein F**, **Vappiani J**, **Sevin DC**, **Tischer C**, **Zinn N**, **Eley JD**, **Gudmann NS**, **Muley T**, **Winter H**, **Fisher AJ**, **Nanthakumar CB**, **Bergamini G**, **Pepperkok R.** An integrated multiomic and quantitative label-free microscopy-based approach to study pro-fibrotic signalling in ex vivo human precision-cut lung slices. *Eur Respir J* 58: 2000221, 2021. doi:10.1183/13993003.00221-2020.
52. **Liu G**, **Särén L**, **Douglasson H**, **Zhou X-H**, **Åberg PM**, **Ollerstam A**, **Betts CJ**, **Balogh Sivars K.** Precision cut lung slices: an ex vivo model for assessing the impact of immunomodulatory therapeutics on lung immune responses. *Arch Toxicol* 95: 2871–2877, 2021. doi:10.1007/s00204-021-03096-y.
53. **Singer BD**, **Mock JR**, **D'Alessio FR**, **Aggarwal NR**, **Mandke P**, **Johnston L**, **Damarla M.** Flow-cytometric method for simultaneous analysis of mouse lung epithelial, endothelial, and hematopoietic lineage cells. *Am J Physiol Lung Cell Mol Physiol* 310: L796–L801, 2016. doi:10.1152/ajplung.00334.2015.
54. **Bantikassegn A**, **Song X**, **Politi K.** Isolation of epithelial, endothelial, and immune cells from lungs of transgenic mice with oncogene-induced lung adenocarcinomas. *Am J Respir Cell Mol Biol* 52: 409–417, 2015. doi:10.1165/rcmb.2014-0312MA.
55. **Reichard A**, **Asosingh K.** Best practices for preparing a single cell suspension from solid tissues for flow cytometry. *Cytometry A* 95: 219–226, 2019. doi:10.1002/cyto.a.23690.
56. **Stegmayr J**, **Alsafadi HN**, **Langwiński W**, **Niroomand A**, **Lindstedt S**, **Leigh ND**, **Wagner DE.** Isolation of high-yield and -quality RNA from human precision-cut lung slices for RNA-sequencing and computational integration with larger patient cohorts. *Am J Physiol Lung Cell Mol Physiol* 320: L232–L240, 2021. doi:10.1152/ajplung.00401.2020.
57. **Mukhopadhyay S**, **Hoidal JR**, **Mukherjee TK.** Role of TNFalpha in pulmonary pathophysiology. *Respir Res* 7: 125, 2006. doi:10.1186/1465-9921-7-125.
58. **Temann A**, **Golovina T**, **Neuhaus V**, **Thompson C**, **Chichester JA**, **Braun A**, **Yusibov V.** Evaluation of inflammatory and immune responses in long-term cultured human precision-cut lung slices. *Hum Vaccin Immunother* 13: 351–358, 2017. doi:10.1080/21645515.2017.1264794.
59. **Martin C**, **Uhlig S**, **Ullrich V.** Videomicroscopy of methacholine-induced contraction of individual airways in precision-cut lung slices. *Eur Respir J* 9: 2479–2487, 1996. doi:10.1183/09031936.96.09122479.
60. **Fiegen RJ**, **Rauen U**, **Hartmann M**, **Decking UK**, **de Groot H.** Decrease of ischemic injury to the isolated perfused rat liver by loop diuretics. *Hepatology* 25: 1425–1431, 1997. doi:10.1002/hep.510250620.
61. **Frank A**, **Rauen U**, **de Groot H.** Protection by glycine against hypoxic injury of rat hepatocytes: inhibition of ion fluxes through nonspecific leaks. *J Hepatol* 32: 58–66, 2000. doi:10.1016/S0168-8278(00)80190-7.
62. **Fukuse T**, **Hirata T**, **Ishikawa S**, **Shoji T**, **Yoshimura T**, **Chen Q**, **Matsukura T**, **Hanaoka N**, **Wada H.** Optimal alveolar oxygen concentration for cold storage of the lung. *Transplantation* 72: 300–304, 2001. doi:10.1097/00007890-200107270-00024.
63. **Bretschneider HJ.** Myocardial protection. *Thorac Cardiovasc Surg* 28: 295–302, 1980. doi:10.1055/s-2007-1022099.
64. **Long LH**, **Halliwell B.** Artefacts in cell culture: α-ketoglutarate can scavenge hydrogen peroxide generated by ascorbate and epigallocatechin gallate in cell culture media. *Biochem Biophys Res Commun* 406: 20–24, 2011. doi:10.1016/j.bbrc.2011.01.091.
65. **Rauen U**, **Klempt S**, **de Groot H.** Histidine-induced injury to cultured liver cells, effects of histidine derivatives and of iron chelators. *Cell Mol Life Sci* 64: 192–205, 2007. doi:10.1007/s00018-006-6456-1.
66. **John MB.** Advances in media for cryopreservation and hypothermic storage (Online), 2005. https://www.semanticscholar.org/paper/Advances-in-Media-for-Cryopreservation-and-Storage-Baust/5f17067dfc94612cd762a634a78120e8e995e7ff.
67. **Koop A**, **Piper HM.** Protection of energy status of hypoxic cardiomyocytes by mild acidosis. *J Mol Cell Cardiol* 24: 55–65, 1992. doi:10.1016/0022-2828(92)91159-3.
68. **Upadhya GA**, **Topp SA**, **Hotchkiss RS**, **Anagli J**, **Strasberg SM.** Effect of cold preservation on intracellular calcium concentration and calpain activity in rat sinusoidal endothelial cells. *Hepatology* 37: 313–323, 2003. doi:10.1053/jhep.2003.50069.
69. **Hochachka PW.** Defense strategies against hypoxia and hypothermia. *Science* 231: 234–241, 1986. doi:10.1126/science.2417316.
70. **Gizewski ER**, **Rauen U**, **Kirsch M**, **Reuters I**, **Diederichs H**, **de Groot H.** Rapid decrease in cellular sodium and chloride content during cold incubation of cultured liver endothelial cells and hepatocytes. *Biochem J* 322: 693–699, 1997. doi:10.1042/bj3220693.
71. **Wahlberg JA**, **Southard JH**, **Belzer FO.** Development of a cold storage solution for pancreas preservation. *Cryobiology* 23: 477–482, 1986. doi:10.1016/0011-2240(86)90056-8.