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Cold-induced injury to lung epithelial cells can be inhibited by iron chelators — implications for lung preservation☆

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Abstract

Objectives: Hypothermia has been shown to lead to cell death via an iron-dependent formation of reactive oxygen species (ROS) in diverse cell types. The susceptibility of lung cells to this injury is unknown — but is of great interest as oxygen is used in lung preservation. Therefore, we studied whether preservation injury to lung epithelial cells can be influenced by iron chelators. **Methods:** A549 lung epithelial cells were preserved at 4 °C for varying periods in either cell culture medium (Dulbecco's Modified Eagle Medium, DMEM), Bretschneider histidine-tryptophan-ketoglutarate (HTK), modified HTK, Celsior, or low potassium dextran (LPD) solution with/without the iron chelators 2,2′-dipyridyl, deferoxamine, or LK 614 and then rewarmed in cell culture medium (for 3 h). Lethal cell injury (lactate dehydrogenase (LDH) release or propidium iodide uptake), metabolic activity, morphological alterations, and lipid peroxidation (thiobarbituric acid-reactive substances, TBARS) were assessed. **Results:** Lung epithelial cells sustained substantial damage after cold storage/rewarming (LDH release: DMEM 66 ± 12% and 97 ± 1%, HTK 73 ± 14% and 97 ± 1%, Celsior 81 ± 16% and 97 ± 1%, LPD 39 ± 14% and 96 ± 1% after 24 h, $n = 7$, and 7 days, $n = 6$, respectively). TBARS were increased. Iron chelators strongly inhibited the damage in all solutions (LDH release after 7 days' cold storage in the presence of 2,2′-dipyridyl: below 8% in all solutions except LPD, LPD 15 ± 4%). This was confirmed using the other iron chelators and the other parameters of injury. **Conclusion:** Hypothermic storage of lung cells leads to iron-dependent oxidative cell injury. These results suggest that the addition of iron chelators to existing or novel preservation solutions might decrease lung preservation injury, and this should now be tested in experimental lung transplantation.

Keywords: Preservation solution; Lung transplantation; Hypothermic storage; Iron chelator; Oxidative cell injury

1. Introduction

Lung transplantation is an established therapy of end-stage lung disease. However, the outcome of this complex procedure is still impaired by relatively high complication rates mostly due to ischemia/reperfusion (I/R) injury, which leads to graft failure with lethal course in up to 30% of the recipients [1]. Although many different studies have been performed, the cellular and molecular mechanisms leading to I/R injury in the lung remain poorly characterized. Therefore, only little improvement has been achieved with the presently used pulmonary preservation solutions, which still differ depending on the transplant center.

Extensive research examining cell damage after lung preservation has shown a complex interaction of different events including production of inflammatory cytokines [2], increased release of reactive oxygen species (ROS) from different cell types [1], loss of epithelial integrity [3], microvascular damage with subsequently increased permeability [4], and cellular infiltration of the tissue. These pathological processes are the results of ischemia, possibly hypothermia (see below) and following reperfusion of the organ, and are likely to be initiated on a cellular level. These pathomechanisms are complex, multifactorial reactions to different stimuli occurring in the time span between organ retrieval and organ implantation with restoration of blood flow. However, compared to other organs, only very limited mechanistic studies have been performed in pulmonary cells up to date.

Current research on the mechanisms of preservation injury in other organs has shown that hypothermia, used as a protective means in organ preservation, leads to cell damage by itself [5]: hypothermia has been shown to lead to an acute increase in intracellular chelatable iron ions, which leads to increased production of highly reactive ROS and subsequently

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to cold-induced apoptosis after rewarming [5–9]. This phenomenon has been shown in different cell types (hepatocytes, renal tubular cells, and different endothelial cells) by different groups, and endangers the cells even more when oxygen is present during hypothermia [8].

This cold-induced cell damage has not yet been examined in the lung; it is of great interest for lung preservation, as the lung is the only organ cold-stored in the presence of oxygen: lungs are preserved mildly inflated with oxygen (in varying concentrations, usually at least 50%). If cold-induced cell damage occurs in lung cells in the same manner as in other organs, it should even be aggravated by the presence of oxygen. This might then explain the high susceptibility of the lung to preservation injury in comparison to other organs. As the deleterious effects of the iron-dependent ROS formation could be inhibited by the addition of iron chelators in other cells, this component of preservation injury is potentially preventable.

It was, therefore, the aim of this study to investigate: (1) whether cold-induced cell damage occurs in lung epithelial cells in different preservation solutions and (2) whether it can be prevented by the addition of iron chelators.

2. Material and methods

2.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM) was obtained from Gibco BRL Life Technologies (Karlsruhe, Germany) and fetal bovine serum, 2,2′-dipyridyl, H33342, propidium iodide, and resazurin were obtained from Sigma Aldrich (Taufkirchen, Germany). Deferoxamine mesylate (Desferal®) was obtained from Novartis Pharma (Nuremberg, Germany) and low potassium dextran (LPD) solution (Perfadex®) was acquired from Carinopharm GmbH (Bonn, Germany). Histidine-tryptophan-ketoglutarate (HTK) solution (Custodiol) and a new, modified HTK solution, N46 (the base solution of new Custodiol-N), as well as the new iron chelator LK 614, were kindly provided by Dr F. Köhler Chemie (Bensheim, Germany). 1,1,3,3-Tetramethoxy-propane was obtained from Fluka, Neu-Ulm, Germany. All other chemicals used were purchased from Merck (Darmstadt, Germany) and gas mixtures were obtained from Messer Griesheim (Krefeld, Germany).

2.2. Cell culture

A549 cells, a human pulmonary epithelial cell line established from lung carcinoma cells, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin (100 U ml^{-1} and 100 μg ml^{-1}, respectively). Cells were maintained in a humidified atmosphere at 37 °C and 8% CO_2 and were subcultured by trypsinization. For the experiments, the cells were split 1:5, seeded onto 12.5-cm^2 or 25-cm^2 culture flasks or six-well and 12-well cell culture plates (BD Biosciences, Heidelberg, Germany), as needed, and used as confluent monolayer cultures on day 5 after subcultivation.

2.3. Experimental procedures

At the beginning of the experiments, the cells were washed with Hanks' balanced salt solution (HBSS, 37 °C) and then covered with cell culture medium or the preservation solutions at room temperature. To part of the incubations, 2,2′-dipyridyl (100 μM), deferoxamine (1 mM), or LK 614 (1 mM) were added. All cells were then incubated at 4 °C for 24 h, or 7, or 21 days. The incubations were performed in an atmosphere of 95% air/5% CO_2 (cell culture medium; for cold incubations, these cell culture flasks/plates were placed in airtight vessels that were flushed with the gas mixture) or under exposure to room air (preservation solutions). After 24 h, or 7, or 21 days of cold incubation, the cells were washed with cold HBSS, covered with cold cell culture medium and rewarmed to 37 °C for 3 h in an incubator containing a humidified atmosphere of 92% air/8% CO_2. The iron chelators were used as follows: all five solutions (DMEM, HTK, N46, Celsior, and LPD) were used with and without 2,2′-DPD in all assays and for all time points. To show that the effects of 2,2′-DPD were not specific for this compound but a general iron chelator effect, deferoxamine was, in addition, used for the first three solutions (DMEM, HTK, and N46) for the 24 h and the 7-day series, and LK 614 was used in N46 for these two series, giving the following summary of conditions for the 24 h and the 7 days series:

(1) DMEM, DMEM + 2,2′-DPD, DMEM + deferoxamine;
(2) HTK, HTK + 2,2′-DPD, HTK + deferoxamine;
(3) N46, N46 + 2,2′-DPD, N46 + deferoxamine, N46 + LK 614;
(4) Celsior, Celsior + 2,2′-DPD; and
(5) LPD, LPD + 2,2′-DPD.

2.4. Assays

2.4.1. Lactate dehydrogenase (LDH) release

Extracellular, that is, released, and intracellular lactate dehydrogenase (LDH) activity were measured using a standard assay, and released LDH activity was given as a percentage of total LDH activity [7].

2.4.2. Alamar Blue (resazurin reduction) assay

The Alamar Blue (resazurin reduction) assay was performed as described in reference [19]. For this assay, cells were cultured in six-well culture plates. Control cells (time zero), and cells exposed to hypothermia (4 °C) in the different solutions, with or without additives, and then rewarmed for 3 h, were carefully washed with HBSS, and warm HBSS supplemented with 10 mM glucose was added. Cells were equilibrated for 15 min at 37 °C; then, resazurin was added at a final concentration of 40 μM. The reduction of resazurin to the fluorescent resorufin was followed continuously at $\lambda_{exc.}$ = 560 nm, $\lambda_{em.}$ = 590 nm for 8 min (37 °C) using a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany). The rate of fluorescence increase (i.e., resazurin reduction) for cells exposed to experimental conditions is given as a percentage of the rate

of fluorescence increase in control cells (in which the assay was performed at time zero).

2.4.3. Thiobarbituric acid-reactive substances

Thiobarbituric acid-reactive substances (TBARS) were determined in the supernatant incubation solution after cold incubation, as described previously [7]; 1,1,3,3-tetramethoxy-propane was used as a standard.

2.4.4. Assessment of cellular morphology

Cellular morphology was assessed by phase contrast microscopy using a Zeiss IM 35 microscope (Zeiss, Oberkochen, Germany) or an Eclipse Ti-U microscope (Nikon, Düsseldorf, Germany).

2.4.5. Assessment of propidium iodide uptake

Nuclei of cells grown on glass coverslips (coated with collagen) were double stained with the DNA-binding fluorochrome H33342 (1 μg ml^{-1}) and the membrane-impermeant DNA-binding fluorochrome propidium iodide (5 μg ml^{-1}), as described previously [7]. Thereafter, cells were assessed using a Zeiss Axiovert 135 TV microscope or an Eclipse Ti-U microscope using the 4′,6-diamino-2-phenylindole (DAPI) and tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC) filter sets. The overlays of both micrographs are shown.

2.5. Statistics

All experiments were performed in duplicate and repeated to a total of 7 times for the 24-h cold-storage series, to a total of 6 times for the 7 days series and to a total of 4 times for the 3-week series. Data are expressed as means ± SD. Comparisons between the different groups (last time point of each series) were performed by an exact non-parametric test (Wilcoxon signed-rank test). A p value of <0.05 was considered significant.

3. Results

3.1. Cold-induced injury of lung epithelial cells

When cultured A549 lung epithelial cells were stored at 4 °C in cell culture medium under aerobic conditions for 24 h, marked cell injury occurred, as demonstrated by LDH release (Fig. 1(A)). During subsequent rewarming, injury increased slightly. Warm control cells showed negligible injury (LDH release: 5 ± 1% after 27 h). Injury as after cold incubation in cell culture medium was observed after 24 h of cold incubation in HTK solution (Fig. 1(B)) or after cold incubation in the new preservation solution N46 (Custodiol-N base solution) or in Celsior solution (Fig. 2); after cold storage in these preservation solutions, there was also some further increase in the injury during rewarming in cell culture medium (Figs. 1(B) and 2). Only after cold incubation in LPD solution, was the injury slightly lower than in cell culture medium, but it still affected more than 35% of cells (Fig. 2). This marked cold-induced cell injury in the organ preservation solutions could be confirmed by propidium iodide uptake (results not shown). In line with the marked injury, metabolic activity of cultured A549 cells was drastically decreased after

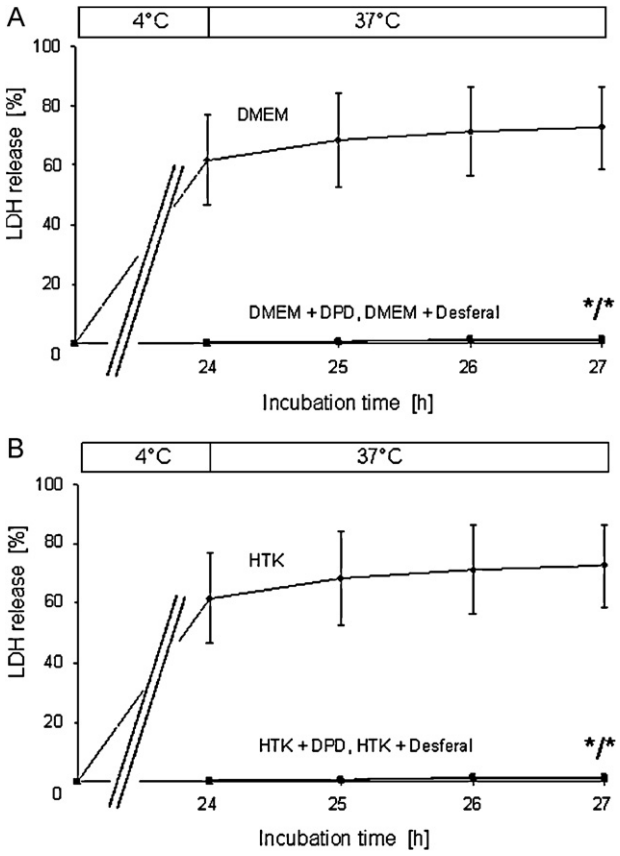

Fig. 1. Inhibition of cold-induced injury of A549 lung cells by iron chelators. Cultured A549 cells were incubated in cell culture medium (Dulbecco's Modified Eagle Medium, DMEM; A) or the preservation solution histidine-tryptophan-ketoglutarate solution (HTK; B) with or without iron chelators at 4 °C for 24 h and then returned to normal cell culture conditions at 37 °C. The iron chelators 2,2′-dipyridyl (DPD, 100 μM) or deferoxamine (Desferal, 1 mM) were added to the cell culture medium or the preservation solution immediately before cold incubation. The occurrence of cell injury was assessed by the release of lactate dehydrogenase (LDH). Values shown represent means ± SD of 7 experiments. (*) Significantly different from the respective incubation without iron chelator, $p < 0.05$.

24 h of cold storage in HTK, N46, and Celsior solutions (Fig. 3). After cold storage in cell culture medium or in LPD solution, metabolic activity was also markedly decreased, but in contrast to the other three solutions, some residual 25–50% of activity was maintained.

3.2. Inhibition of cold-induced injury by iron chelators

The addition of the iron chelator 2,2′-dipyridyl completely inhibited the occurrence of cold-induced cell injury, with values of LDH release after rewarming remaining below 2% in cultures cold exposed in cell culture medium (Fig. 1(A)) as well as in HTK solution (Fig. 1(B)). Similar protection by 2,2′-dipyridyl could be seen in the other preservation solutions used (Fig. 2). Furthermore, the iron chelator deferoxamine (Desferal; Fig. 1(A)/(B)) provided protection similar to 2,2′-dipyridyl. Similarly, LK 614, a new iron chelator used in Custodiol-N, provided complete protection when added to N46 (LDH release: 1 ± 1% after 3 h of rewarming).

The protective effect of the iron chelators could be confirmed using a different parameter of cell injury, that is,

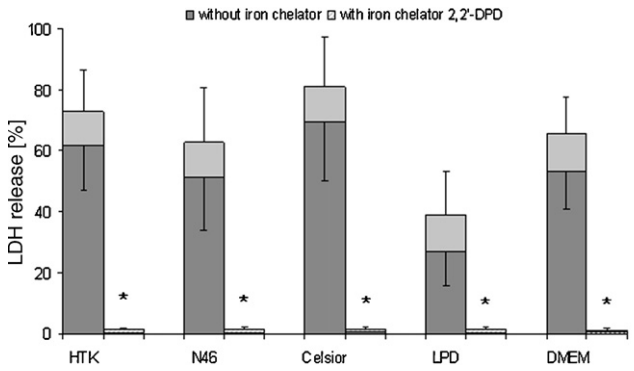

Fig. 2. Inhibition of cold-induced injury of A549 lung cells by the iron chelator 2,2′-dipyridyl (DPD) in different solutions. Cultured A549 cells were incubated in cell culture medium (Dulbecco's Modified Eagle Medium, DMEM) or in different types of preservation solutions [histidine-tryptophan-ketoglutarate solution (HTK), the new solution N46, Celsior or low-potassium-dextran solution (LPD)] at 4 °C for 24 h (dark grey part of the bars) and then returned to normal cell culture conditions at 37 °C (light grey part of the bars). The iron chelator 2,2′-dipyridyl (DPD, 100 μM) was added to the cell culture medium or the preservation solution immediately before cold incubation (hatched bars). The occurrence of cell injury was assessed by the release of lactate dehydrogenase (LDH). Values shown represent means ± SD of 7 experiments. (*) Significantly different from the respective incubation without iron chelator, $p < 0.05$.

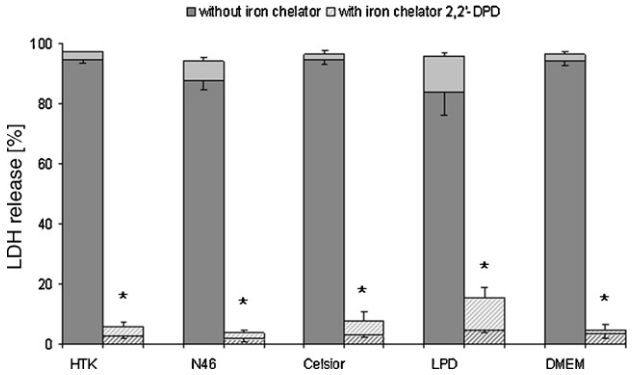

Fig. 4. Long-term inhibition (7 days) of cold-induced injury of A549 lung cells by the iron chelator 2,2′-dipyridyl (DPD). Cultured A549 cells were incubated in cell culture medium (Dulbecco's Modified Eagle Medium, DMEM) or in the preservation solutions histidine-tryptophan-ketoglutarate solution (HTK), the new solution N46, Celsior and low-potassium-dextran solution (LPD) with or without the iron chelator 2,2′-dipyridyl (DPD) at 4 °C for 7 days (dark grey part of the bars) and then returned to normal cell culture conditions at 37 °C (light grey part of the bars). The iron chelator 2,2′-dipyridyl (DPD, 100 μM) was added to the cell culture medium or the preservation solution immediately before cold incubation (hatched bars). The occurrence of cell injury was assessed by the release of lactate dehydrogenase (LDH). Values shown represent means ± SD of 6 experiments. (*) Significantly different from the respective incubation without iron chelator, $p < 0.05$.

propidium iodide uptake (data not shown). Furthermore, the addition of the iron chelators 2,2′-dipyridyl, or deferoxamine, as well as LK 614 (data not shown) resulted in almost complete preservation of the metabolic activity (Fig. 3).

3.3. Long-term cold storage of A549 cells in the presence of iron chelators

Practically complete protection by the iron chelator 2,2′-dipyridyl could be observed even after 7 days of cold storage

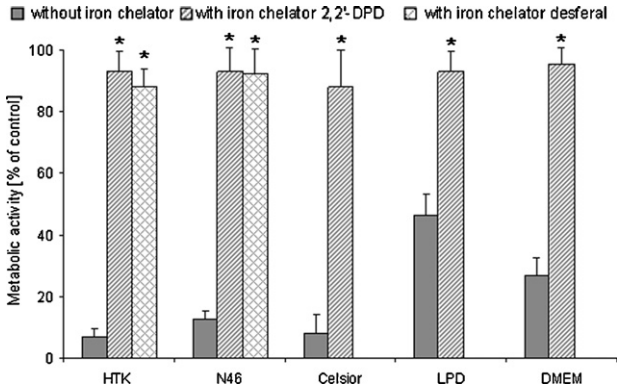

Fig. 3. Preservation of metabolic activity by iron chelators. Cultured A549 cells were incubated in cell culture medium (Dulbecco's Modified Eagle Medium, DMEM) or in the preservation solutions histidine-tryptophan-ketoglutarate solution (HTK), the new solution N46, Celsior or low-potassium-dextran solution (LPD) with or without iron chelators at 4 °C for 24 h and then returned to normal cell culture conditions at 37 °C. The iron chelators 2,2′-dipyridyl (DPD, 100 μM) or deferoxamine (1 mM, only used in HTK and N46) were added to the cell culture medium or the preservation solution immediately before cold incubation. Metabolic activity was assessed by the Alamar Blue (resazurin reduction) assay and is displayed as percentage of the value of control cells control cells (cells not exposed to hypothermia). Values shown represent means ± SD of 7 experiments. (*) Significantly different from the respective incubation without iron chelator, $p < 0.05$.

at 4 °C, decreasing LDH release after rewarming from >90% to <8% in cell culture medium and in all preservation solutions used, except for LPD solution where LDH release could be decreased to only 15 ± 4% (Fig. 4). The addition of deferoxamine or LK 614 to HTK solution or N46 base solution led to similar protection (data not shown).

Preservation of cell integrity was underlined by the morphological assessment, as shown by the example of A549 cells incubated for 7 days at 4 °C in cell culture medium and rewarmed at 37 °C for 3 h where cells cold stored without iron chelator showed marked destruction (Fig. 5(B) and (C)), whereas iron-chelator-protected cells (Desferal, 1 mM) showed retraction after cold storage (Fig. 5(D)) but restoration to almost normal morphology during rewarming (Fig. 5(E)) when compared with control (Fig. 5(A)). Fluorescence microscopy results were even more impressive, showing mostly propidium iodide-positive, that is, dead cells (stained red) after cold storage in the absence of iron chelators (Fig. 6(A) and (C)), but hardly any dead cells after cold storage in the presence of the iron chelator deferoxamine (Fig. 6(B) and (D)).

The protective effect of the iron chelator 2,2′-dipyridyl could still be seen after 21 days of cold storage: addition of the iron chelator 2,2′-dipyridyl significantly decreased cell damage after cold storage in all preservation solutions, most markedly in N46 and HTK solution where LDH release at the end of the cold storage itself remained below 5% (Table 1). Cell damage in Celsior, LPD solution, or in cell culture medium with the iron chelator was higher but still markedly below values in the absence of the iron chelator. Cell damage substantially increased during rewarming after this extensive cold incubation period, so that LDH release exceeded 80% after 3 h of rewarming in cells cold stored in HTK solution, Celsior, and LPD plus the iron chelator. In N46 + 2,2′-dipyridyl and in cell culture medium + 2,2′-dipyridyl, however, LDH release still remained below 65% even after rewarming, so

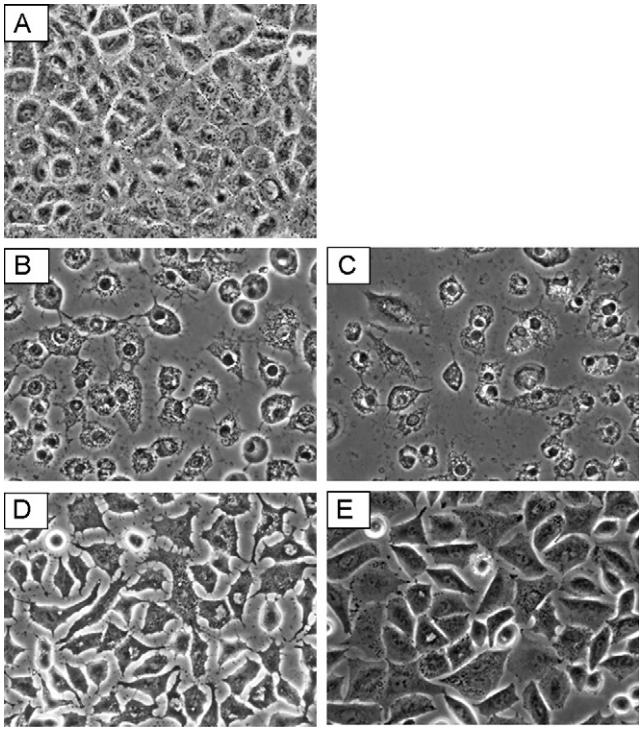

Fig. 5. Morphological alterations of cultured human pulmonary epithelial cells exposed to hypothermia/rewarming. A549 cells were incubated in cell culture medium at 4 °C for 7 days and then returned to normal cell culture conditions at 37 °C. The morphology was assessed by phase contrast microscopy (original magnification 400×). Panel (A) shows the cells before hypothermia as control. On panels (B) and (C) the cells were stored in normal cell culture medium during hypothermia, on panels (D) and (E) the iron chelator deferoxamine (1 mM) was added before cold storage. (B) and (D) show the cells directly after 7 days of hypothermia, (C) and (E) after 3 h of rewarming. Note that cells displayed major damage without the presence of the chelator (B and C) and did not expand again after rewarming (C) whereas the cells only retracted (D) during hypothermia but were able to reextend after rewarming (E) if cold incubation was performed in the presence of the iron chelators.

that, in these solutions, the injury after 21 days of cold storage plus 3 h of rewarming in the presence of an iron chelator was roughly equivalent to the extent of the injury after 24 h of cold storage plus rewarming in the absence of an iron chelator.

3.4. Warm toxicity of preservation solutions

Twenty-seven hours of warm (37 °C) aerobic incubation of A549 cells in LPD, Celsior solution, or cell culture medium led to a markedly increased LDH release in the cells incubated in LPD solution in contrast to Celsior and cell culture medium (LPD: 74 ± 2%; Celsior: 8 ± 1%; and cell culture medium: 4 ± 1%).

3.5. Cold-induced lipid peroxidation and its inhibition by iron chelators

Twenty-four hours of cold storage of A549 cells led to marked lipid peroxidation in all solutions used (Fig. 7). The addition of the iron chelator 2,2′-dipyridyl during cold incubation significantly decreased lipid peroxidation in all solutions.

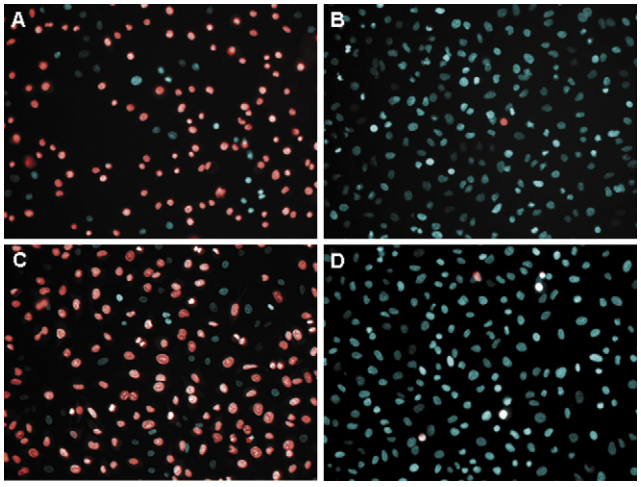

Fig. 6. Assessment of cell death of cultured human pulmonary epithelial cells exposed to hypothermia/rewarming. A549 cells were incubated in DMEM culture medium (A and B) or N46 solution (C and D) at 4 °C for 7 days and then rewarmed under normal cell culture conditions for 3 h at 37 °C. To both solutions the iron chelator deferoxamine (1 mM) was added (panels B and D). Cellular death was assessed by fluorescence microscopy (λ_{exc} = 340–380 nm; λ_{em} = 435–485 nm for H33342 and λ_{exc} = 540 ± 12.5 nm, λ_{em} = 605 ± 27.5 nm for propidium iodide) after double-staining the cells with the membrane-permeable DNA-binding fluorochrome H33342 (1 μg ml^{-1}), staining all nuclei (blue fluorescence), and the DNA-binding fluorochrome propidium iodide (5 μg ml^{-1}), which only stains nuclei of dead cells (red fluorescence). Addition of the iron chelator inhibited cell injury as displayed by the lack of red staining of the nuclei in panels (B) and (D).

4. Discussion

In this study, we observed a prominent, iron-dependent, cold-induced cell injury in cultured lung epithelial cells. This injury occurred in organ preservation solutions as well as in cell culture medium and might represent a major, but easily preventable, component of lung preservation injury.

Hypothermia, usually used as a protective means in organ preservation, undoubtedly triggered the injury as the injury was only observed after cold (4 °C) but not after warm incubation in cell culture medium. The injury was not due to hypoxia, as the model used allows for aerobic conditions. The cold-induced cell injury, confirmed using several parameters

Table 1. Long-term inhibition (21 days) of cold-induced injury of A549 lung cells by the iron chelator 2,2′-dipyridyl (DPD).

Preservation solution	LDH release (%)	
	Without DPD	With DPD
HTK	95 ± 1	3 ± 5*
N46	84 ± 4	2 ± 4*
Celsior	95 ± 1	27 ± 3*
LPD	87 ± 2	16 ± 4*
DMEM	97 ± 1	39 ± 5*

Cultured A549 cells were incubated in cell culture medium (Dulbecco's Modified Eagle Medium, DMEM) or in the preservation solutions histidine–tryptophan–ketoglutarate solution (HTK), the new solution N46, Celsior or low-potassium-dextran solution (LPD) with or without iron chelator at 4 °C for 21 days. The iron chelator 2,2′-dipyridyl (DPD, 100 μM) was added to the cell culture medium or the preservation solution immediately before cold incubation. The occurrence of cell injury was assessed by the release of lactate dehydrogenase (LDH). Values shown represent means ± SD of 4 experiments.

* Significantly different from the respective incubation without iron chelator, $p < 0.05$.

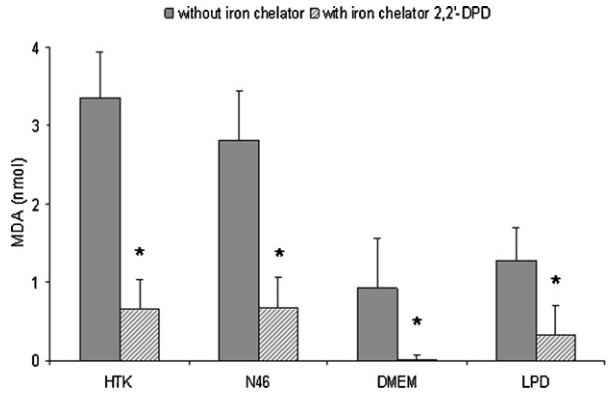

Fig. 7. Inhibition of lipid peroxidation by the iron chelator 2,2′-dipyridyl. A549 cells were incubated in cell culture medium (Dulbecco's Modified Eagle Medium, DMEM) and in the preservation solutions histidine-tryptophan-ketoglutarate (HTK), the new solution N46 and low-potassium-dextran solution (LPD) at 4 °C for 24 h. The iron chelator 2,2′-dipyridyl (DPD, 100 μM) was added to the cell culture medium or the preservation solution immediately before cold incubation. Lipid peroxidation was assessed by determination of thiobarbituric acid-reactive substances (TBARS). Values shown represent means ± SD of 6 experiments. (*) Significantly different from the respective incubation without iron chelator, $p < 0.05$.

of cell injury or function (LDH release, morphology, propidium iodide uptake, and resazurin reduction; Figs. 1–6, Table 1), was iron-dependent (i.e., iron-chelator inhibitable) and was accompanied by lipid peroxidation (Fig. 7), suggesting its mediation by ROS. Thus, this type of injury, described for the first time for lung epithelial cells, appears to be similar to the cold-induced injury of cells of other transplantable organs [5–7,9].

The mechanism of cold-induced cell injury, as described in other cell types, namely hepatocytes, renal tubular cells, and liver endothelial cells involves a marked increase in the cellular chelatable iron pool, that is, in the strictly regulated cellular pool of redox-active iron ions, which are able to convert ROS of low toxicity into highly reactive species, such as the hydroxyl radical or iron–oxygen species [5–8]. The increase in cellular chelatable iron ions has been described to occur rapidly after initiation of cold incubation in hepatocytes and on a somewhat longer timescale in renal tubular cells. If oxygen is present, this increased availability of redox-active iron ions leads to marked lipid peroxidation and to a mitochondrial permeability transition or mitochondrial ultracondensation, both mitochondrial alterations known to be involved in apoptotic pathways, and both being inhibited by the use of iron chelators [5,10,11].

Hypothermic injury has long been thought to be caused by alterations of cellular ion homeostasis and/or dysregulation of metabolism. Classically, cell hypothermic injury is attributed to an intracellular accumulation of sodium, accompanied by an accumulation of chloride, and followed by an influx of water [12]. The accumulation of sodium is usually attributed to an inhibition of the Na^+/K^+-adenosine triphosphatase (Na^+/K^+-ATPase) at low temperature [12,13], and the cellular edema caused by these alterations has long been thought to be the cause of hypothermic injury. Similar mechanisms in the cellular calcium homeostasis, with decreased efflux by the (cold-inhibited) Ca^{2+}-ATPase and secondary to the alterations in cellular sodium homeostasis, have been suggested to lead to an increase in the cytosolic calcium concentration with the potential sequelae of activation of calcium-dependent hydrolases, especially of phospholipases [12]. However, the role of these mechanisms is challenged by the results presented here, as: (1) use of cell culture medium (with physiological extracellular sodium, calcium, and chloride concentrations) did not cause higher injury than the use of sodium-, calcium- and chloride-poor organ preservation solutions, (2) the use of solutions designed to counteract these changes (organ preservation solutions, see later) did not affect the severity of cold-induced injury in this model, and (3) iron chelators provided complete protection, even after very extensive cold-storage periods, such as 1 week. Similar findings, that is, no evidence for a role of sodium or calcium homeostasis in pure cold-induced cell injury (as opposed to combined hypoxic/hypothermic injury), have been reported for hepatocytes, liver endothelial cells, and renal tubular cells [8].

Present preservation solutions are based on the above classical concept of a major role for sodium and calcium in the pathogenesis of hypothermic injury, and do not provide efficient protection against the cold-induced injury originating from the iron-dependent lipid peroxidation. Iron-dependent cold-induced injury occurred in all preservation solutions used in the current study, representing the solutions used most commonly worldwide.

Thus, these results suggest the incorporation of an iron chelator into preservation solutions. Only LPD solution showed marginal superiority, possibly due to its component dextran, for which additional properties have been described aside its oncotic effect; it is even discussed whether dextran plays a role in reducing the production of reactive oxygen species [14]. However, even LPD solution could not offer the protection observed by the addition of iron chelators. In the presence of iron chelators, LPD solution appeared to be inferior to other solutions (Fig. 4). This might be due to a certain toxicity of the LPD solution, which also became evident in the warm control experiments, and which has also been described for other preservation solutions [15].

Cold-induced cell injury could be a major component of preservation injury in the setting of lung transplantation, as it is the only organ, which is cold stored in the presence of oxygen (inflated with air/oxygen). Thus, lung epithelial cells are, on the one hand, unlikely to suffer hypoxia during lung storage (although the lung is ischemic). On the other hand, the presence of oxygen fosters the iron-dependent formation of ROS, and cold-induced cell injury has also been shown to be aggravated by an increased availability of oxygen [6]. In line with these mechanistical considerations, lung preservation studies have shown the role of oxygen concentration in the explanted lung, with significantly less ROS production in lungs inflated with 5% oxygen, instead of 21% or 100% [16].

These findings have an important implication concerning the presently used preservation protocols, as none of the solutions used clinically account for iron-dependent cold-induced injury of cells. Addition of iron chelators should be strongly considered when contemplating the preservation of the cell integrity as well as the cell function, especially in the case of the lung in which hypothermic cell injury is likely to play a greater role and ischemic injury a lesser role than in other organs. Preservation of cell integrity and function could

lead to a better 'whole' organ preservation, and therefore lead to a better organ function after transplantation, reducing preservation injury (most commonly called 'I/R injury'), which is still a major problem, especially in lung transplantation [17,18].

The question is which base solution should be supplemented by iron chelators, especially as the widely used LPD solution appeared to be inferior to other solutions in the presence of iron chelators (Fig. 4, Table 1). Recently, our group developed a preservation solution based on the initial HTK solution, modified on the basis of diverse experimental studies. In this solution, Custodiol-N, histidine was partially replaced by *N*-acetylhistidine [19] to avoid a certain toxicity of histidine while keeping its excellent buffering capacity, glycine and alanine were added to inhibit hypoxic cell injury, and the iron chelators deferoxamine and LK 614 were incorporated to inhibit hypothermic injury [20]. Here, the base solution of Custodiol-N (N46) already showed equivalence, if not superiority, to the other preservation solutions. Similarly, precursor solutions of the base solution also showed equivalence or superiority, dependent on the parameter considered, to HTK solution, in a study with isolated rat aortic rings [21]. The protective effects of the new base solution in comparison to HTK solution are, in both models, likely to be due to the partial replacement of histidine; however, protection by the altered base solution is (evidently) relatively small in cases where the iron-dependent injury predominates. In the presence of iron chelators, and especially with long cold-storage times, in the current series, the new solution proved to be excellent and, if anything, superior to other preservation solutions, even if iron chelators were also added to these solutions (Figs. 4 and 6, Table 1). For use in organ preservation, addition of a combination of iron chelators (deferoxamine and LK 614) is envisaged in Custodiol-N. Deferoxamine is a hydrophilic iron chelator with high molecular weight, which has been shown to decrease the formation of hydroxyl radicals or iron-oxygen species [22]. However, due to its size and hydrophilicity, cell penetration is limited. Therefore, LK 614, a smaller and more lipophilic iron chelator, which is more membrane permeable, will be used in the preservation solution in addition to deferoxamine to ensure intracellular availability of an iron chelator. LK 614 also showed strong protection in the current study. The new preservation solution, as base solution (N46) and supplemented by iron chelators (Custodiol-N), showed largely improved liver and heart preservation in different experimental studies [20,23,24], and will, based on the encouraging results described here, soon be tested in an experimental model of lung transplantation.

A549 cells are lung epithelial cells widely used as experimental model and have also been described as a cell model for the simulation of the clinical process of lung transplantation with aerobic, hypothermic ischemia with subsequent warm reperfusion mimicking the current clinical practice of lung preservation and transplantation [25]. We, therefore, chose this cell line to study the susceptibility of lung cells to cold-induced cell injury, the mechanism of this injury, and the effects of organ preservation solutions. Cultured cells are excellent for screening purposes and for mechanistic studies; however, results obtained in cell cultures have to be confirmed in animal models before transfer to the clinical situation can be envisaged. These experiments are currently ongoing.

In conclusion, we here demonstrated that lung epithelial cells suffer an iron-dependent cold-induced cell injury similar to the injury described in other cell types. This injury could be strongly inhibited by the use of iron chelators, which, therefore, are likely to improve lung preservation, and will now be tested in a lung transplantation model.

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