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# Evaluation of the protection exerted by *Pisum sativum* Ferredoxin-NADP(H) Reductase against injury induced by hypothermia on Cos-7 cells ☆

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ABSTRACT

Hypothermia is employed as a method to diminish metabolism rates and preserve tissues and cells. However, low temperatures constitute a stress that produces biochemical changes whose extension depends on the duration and degree of cold exposure and is manifested when physiological temperature is restored. For many cellular types, cold induces an oxidative stress that is dependent on the elevation of intracellular iron, damages macromolecules, and is prevented by the addition of iron chelators. *Pisum sativum* Ferredoxin-NADP(H) Reductase (FNR) has been implicated in protection from injury mediated by intracellular iron increase and successfully used to reduce oxidative damage on bacterial, plant and mammalian systems. In this work, FNR was expressed in Cos-7 cells; then, they were submitted to cold incubation and iron overload to ascertain whether this enzyme was capable of diminishing the harm produced by these challenges. Contrary to expected, FNR was not protective and even exacerbated the damage under certain circumstances. It was also found that the injury induced by hypothermia in Cos-7 cells presented both iron-dependent and iron-independent components of damage when cells were actively dividing but only iron-independent component when cells were in an arrested state. This is in agreement with previous findings which showed that iron-dependent damage is also an energy-dependent process.



## Introduction

The protective effects of low temperatures have been known and explored since ancient times and reported as early as 1683 [50]. The scientific bases of the cellular death after an ischemic period and its improvement by hypothermia began to be comprehended some decades ago. The rational for this is to submit organs, tissues and cells to low temperatures to dramatically diminish the metabolism in a reversible manner by physicochemical mechanisms and achieve an arrested status of enzymatic and non-enzymatic cellular processes. For cells of non-poikilothermic animals, low temperatures constitute a stress that must be withstood and whose effects depend on the magnitude and time of applied cold [50]. Nevertheless, despite hypothermia at 0 °C being a widely used method to protect cells and tissues from deleterious processes, in some cellular types – like hepatocytes and liver endothelial cells – it produces a vast damage mediated by reactive oxygen species (ROS)[3] [37–39,46].

ROS participation in cold preservation injury was proved by the considerable lipid peroxidation produced and the strong inhibition of the damage achieved through imposition of hypoxia and incubation with some antioxidant compounds – free radical scavengers (5,5-dimethyl-1-pyrroline *N*-oxide: DMPO), lipophilic antioxidants (buthylated hydroxytoluene: BHT), iron chelators (deferoxamine: DFO), and hydroxyl radical scavengers (dimethyl sulfoxide: $Me_2SO$) [30,37–39,46,52,54].

Additional experiments with hepatocytes and liver endothelial cells have shown that hypothermic injury – mediated by ROS – is produced, at least in part, by alterations in the homeostasis of cellular iron [30,44]. The major part of intracellular iron is strongly

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[3] *Abbreviations used:* ROS, reactive oxygen species; FNR, Ferredoxin-NADP(H) Reductase; DFO, deferoxamine; $Me_2SO$, dimethyl sulfoxide; $OH^{\cdot -}$, hydroxyl radical; $H_2O_2$, hydrogen peroxide; mUW, modified University of Wisconsin solution; MV, methyl viologen; HTK, Bretschneider solution; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NBT, nitroblue tetrazolium chloride; DAB, diaminebenzidine tetrahydrochloride; MDA, malondialdehyde; 8HQ, 8-hydroxyquinoline.

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bound to different proteins (ferritin or hemeproteins) which are distributed in a variety of sub-cellular compartments. However, a small portion (0.2–3%) of cellular iron represents the "iron in transit", which forms complexes with low molecular weight organic compounds of relatively low affinity (citric acid, amino acids, sugars, ascorbate, ADP, ATP and other nucleotides) or is weakly associated to membrane proteins or lipids [33,44]. This iron fraction is metabolically and catalytically active and commonly known as "labile iron pool" or "chelatable iron pool" [17]. Differently from storage or protein bound iron, this iron in transit has a great cytotoxic potential. This cytotoxicity is mainly related to the fact that iron can catalyze the formation of hydroxyl radicals ($OH^{\cdot -}$) from hydrogen peroxide ($H_2O_2$) or iron-oxygen species [36]. After hepatocytes were incubated for different periods of time under hypothermia, it was observed a fast increase in the chelatable iron pool levels without an increase in the production of hydrogen peroxide and superoxide. This made clear the main role of hydroxyl radical and ferrilum species in this kind of cellular injury [44].

Hypothermia is generally followed by restoration of physiological temperature – rewarming. When liver cells were rewarmed after sub-lethal periods of cold preservation, they displayed clear apoptotic signals. Although the apoptosis is manifested during rewarming, its extent depends on the length of incubation at 0 °C, which suggests that the biochemical alterations provoked by cold are a prerequisite for apoptosis to occur [45]. Similar evidence has been observed *in vitro* in renal and cardiac grafts and renal cells, which could indicate that the same universal phenomenon occurs in different mammalian cells [11,15,26,32].

We have previously demonstrated that the expression of the *Pisum sativum* enzyme Ferredoxin-NADP(H) Reductase (FNR, E.C. 1.18.1.2) conferred to cold preserved rat isolated hepatocytes an augmented ability to engraft in liver parenchyma of receptor animals when they were used for hepato-cellular transplantation [28]. Similarly, we found that this enzyme protected Cos-7 cells when they were exposed to hydrogen peroxide but not when exposed to methyl viologen (MV) *in vitro* [27]. In these works, FNR was directed to mitochondria since this organelle is an early target of hypothermia/rewarming damage that is considered to be triggered by iron elevation from cytosol [7,43]. *P. sativum* FNR was also implicated in the protection of *Escherichia coli* mutant strains lacking the bacterial ferredoxin reductase against methyl viologen exposure. This compound produces an oxidative injury through the formation of $OH^{\cdot -}$ *in situ*, which is caused by an increase in the intracellular iron level [20].

In the current work, we evaluated the capability of *P. sativum* FNR to protect the transfected Cos-7 cells mentioned above from injury provoked by exposure to pure hypothermia – without imposition of hypoxia. Remarkably and contrary to what antecedents suggested, we did not find protection against damage produced by hypothermia or iron in cells that express FNR. Full discussion of the results is presented below.

## Materials and methods

### *Plasmids*

pFNR was constructed from pcDNA3 (Invitrogen #V79020) by the insertion of the mitochondrial import sequence from the mouse ferredoxin reductase gene (GenBank accession #D49920) fused in phase with the mature portion of *P. sativum fnr* gene (GenBank accession #X12446) between the *Hind* III and *Eco* RI sites of the multiple cloning site of the plasmid. The strategy used for cloning has been described previously [27,28].

### *Cell culture conditions and transfections*

Cos-7 cells (ECACC 87021302) were cultured in DMEM/High glucose medium (Sigma D5648) supplemented with 10% fetal bovine serum (FBS, Biotecnológico R.A. Suermer) in a $CO_2$ incubator at 37 °C. Transfections were carried out using jetPEI (Qbiogene)

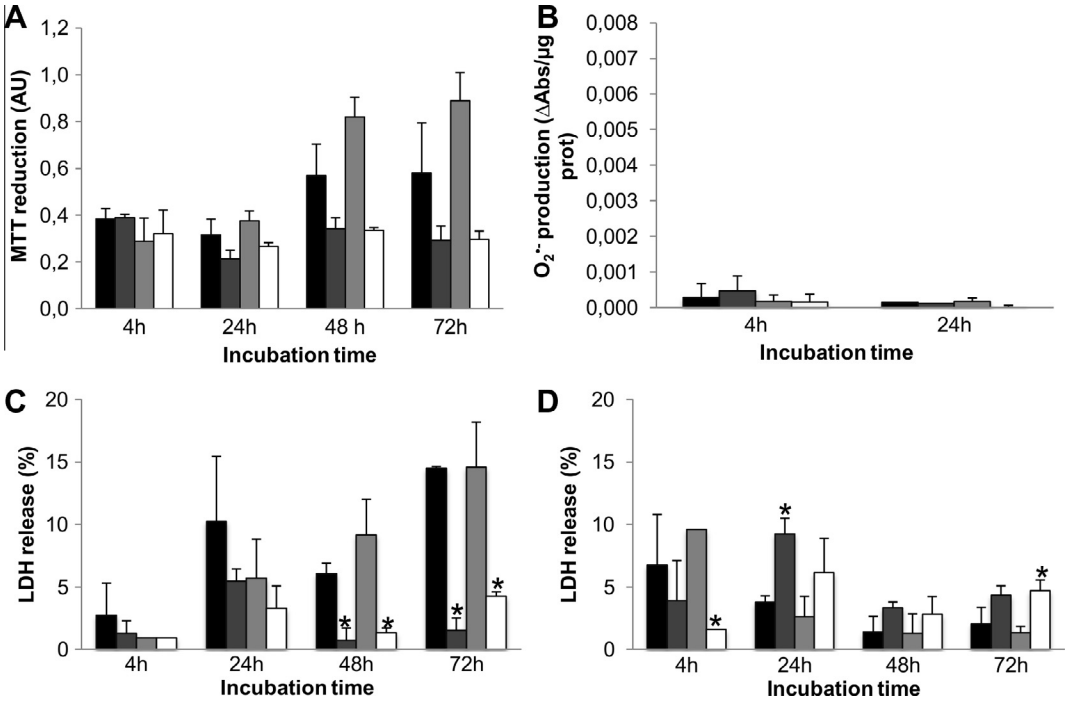


**Fig. 1.** Hypothermia effects after different periods of incubation at 0 °C in mUW solution. (A) Cell viability/growth measured by MTT reduction. The results are expressed in absorbance units at 570 nm. (B) Cell damage after incubation under hypothermia measured by LDH release. (C) Cell damage after rewarming period measured by LDH release. (D) Superoxide anion production measured by NBT reduction assay. The results are expressed in absorbance units at 520 nm per μg of protein. Black and dark gray bars: Cos-7/pcDNA3 cells incubated at 37 °C and preserved at 0 °C, respectively. Light gray and white bars: Cos-7/pFNR cells incubated at 37 °C and preserved at 0 °C, respectively, $n = 3$ experiments, in duplicate. $^*P < 0.05$, different from cells under control condition.

following the manufacturer's instructions and the transfectant selection was done with 0.50 mg/ml G418 (Sigma A1720) as previously reported [27]. Expression and sub-cellular localization of FNR were confirmed by total diaphorase activity increment, and confocal microscopy and Western Blot as formerly published [27]. To conduct the experiments, all lines were used within 25 passage numbers to reduce variations due to cell line characteristic alterations. Cells were seeded the day before the experiments at $3.5 \times 10^4$ cells/cm$^2$ – for exponentially growing phase – and at $10^5$ cells/cm$^2$ – for confluent stationary phase cultures.

*Incubation in hypothermia*

Monolayers were washed with PBS and incubated at 0 °C with a variety of pre-chilled solutions: mUW, HTK (Celsior® type), culture medium (DMEM + FBS) or Krebs Henseleit buffer (K–H), as indicated (compositions are shown in Table S1). The cells were incubated for different periods of time (4–72 h) in an ice bath at 0 °C under an air:$CO_2$ (95:5) atmosphere (inflowed at a constant rate of 30 mL/min during the entire incubation).

*Rewarming*

After incubation in hypothermia, cell monolayers were returned to 37 °C. They were washed with PBS, plates were filled with culture medium and placed in a standard $CO_2$ incubator for 120 min.

*Incubation with iron*

As a positive control for cellular damage, cell monolayers were incubated with a 1:1 complex of $Fe_2Cl_3{\cdot}6H_2O$ and 8-hydroxyquinoline (8HQ, MP Biomedicals 151310) (2.5–15 μM final concentration) in standard culture medium and conditions [16].

*Cell viability and metabolic function*

Lactate Dehydrogenase (LDH) activity was determined by measuring NADH oxidation at $\lambda = 340$ nm in a reaction mixture containing 0.6 mM pyruvate and 0.2 mM NADH in 50 mM potassium phosphate pH 7.50. ΔA/min was monitored for 3 min at 37 °C [23].

For MTT (dimethylthiazolyl diphenyltetrazolium bromide, Sigma M2128) reduction assay, cell monolayers were washed with PBS and culture medium with 0.50 mg/ml MTT was added and incubated for 30 min in a $CO_2$ incubator. After incubation, insoluble violet formazan crystals were dissolved with 3% SDS and 0.03 N HCl in 70% isopropanol and absorbance was read at $\lambda = 570$ nm [10].

*ROS and oxidative damage*

$O_2^{\cdot -}$ levels were measured by two different methods: reduction of Nitroblue tetrazolium chloride (NBT, Sigma N6876) [44] and cytochemical staining with 3,3′-Diaminebenzidine tetrahidrochloride (DAB, Sigma D5637) [49]. To measure NBT reduction, cell monolayers were washed with K-H, and incubated for 2 h in a $CO_2$ incubator with K–H buffer and 1 mg/mL NBT. Then, insoluble formazan crystals were dissolved with 2 M KOH (1 part) and $Me_2SO$ (1.167 parts) separately added and absorbance was measured at $\lambda = 520$ nm. The assay was performed with and without 500 U/ml of Superoxide Dismutase (SOD, Sigma S7571) to distinguish NBT reduction produced by $O_2^{\cdot -}$ from that produced by other cellular activities, in particular, the diaphorase activity of FNR [2]. For DAB staining, cell monolayers were washed with 5% sucrose, 0.5 mM $MgCl_2$, 1 mM sodium azide in 0.1 M HEPES buffer pH 7.20 and incubated for 30 min in the same solution containing 2.5 mM DAB. After incubation, cells were washed in buffer and fixated in 2% glutaraldehyde for 20 min at room temperature.

**Table 1**
Lipid peroxidation. MDA levels in different conditions measured by HPLC.

| Incubation condition | Cos-7/pcDNA3 (μM) | Cos-7/pFNR (μM) |
|---|---|---|
| 37 °C (72 h + rewarming) | 2.42 ± 0.19 | 3.95 ± 0.28[c] |
| mUW[a] (72 h + rewarming) | 2.47 ± 0.28 | 5.20 ± 0.30[c] |
| 37 °C (24 h) | 4.10 ± 0.35 | 4.65 ± 0.19 |
| HTK[b] (24 h) | 0.069 ± 0.004 | 0.026 ± 0.002[c] |
| 5 μM Fe (24 h) | 2.41 ± 0.20 | 2.81 ± 0.17 |

[a] Modified University of Wisconsin solution.
[b] Histidine–Tryptophan–Ketoglutarate solution.
[c] Different from Cos-7/pcDNA3 in the same incubation condition; $n = 3$ experiments.

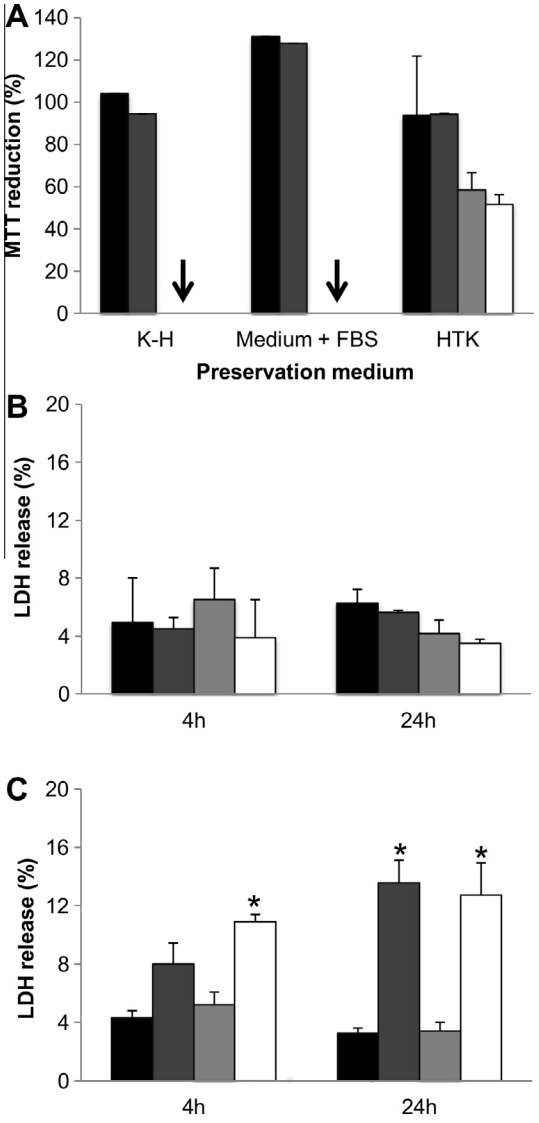


**Fig. 2.** Hypothermia effects after 4 and 24 h of incubation at 0 °C in solutions distinct from mUW. (A) Cell viability/growth after incubation under hypothermia in K–H buffer, culture medium supplemented with 10% FBS and HTK preservation solution measured by MTT reduction assay. The results were expressed in percentage respect to control cells incubated in the same solution and maintained at 37 °C. The arrows indicate total cellular death. (B) Cell damage after incubation under hypothermia in HTK solution measured by LDH release. (C) Cell damage after rewarming period following incubation in HTK at 0 °C measured by LDH release. (A) Black and dark gray bars: Cos-7/pcDNA3 cells incubated for 4 and 24 h, respectively. Light gray and white bars: Cos-7/pFNR cells incubated for 4 and 24 h, respectively; (B and C) Black and dark gray bars: Cos-7/pcDNA3 cells incubated at 37 °C and preserved at 0 °C, respectively. Light gray and white bars: Cos-7/pFNR cells incubated at 37 °C and preserved at 0 °C, respectively, $n = 3$ experiments, in duplicate. *$P < 0.05$, different from cells under control condition.

For the estimation of $OH^{\cdot -}$ levels, cells monolayers were washed with PBS, lysed with 1 volume of distillated water and incubated with 0.05 M EDTA, 0.05 mM ascorbic acid, with or without 1.8 mM 2-deoxy-D-ribose (DR, Sigma F-31170) for 1 h at 37 °C as $OH^{\cdot -}$ breaks deoxy-ribose to produce malondialdehyde (MDA) in these conditions [13,14]. Then, one volume of the reaction mixture was mixed with 1 volume of 10% trichloroacetic acid and 1 volume of 0.375% W/V 2-thiobarbituric acid and incubated for 15–20 min at 100 °C to react with the MDA produced. Absorbance was measured at $\lambda = 532$ nm.

Oxidative damage to lipids was also quantified by MDA and conjugated diene formation. MDA was measured in an HPLC system using a C18 column. Mobile phase consisted of 30 mM $KH_2PO_4$ pH 4.00/methanol (65/35% v/v) at a flow rate of 1.5 mL/min [18]. UV detection was set at $\lambda = 254$ nm. As standard, 1,1,3,3-tetraethoxypropane (TEP, Sigma T9889) was diluted in 0.1 M HCl, boiled for 5 min and diluted to 50 μM MDA ($\varepsilon = 13700\ M^{-1}\ cm^{-1}$ at $\lambda = 254$ nm). Samples and standard were diluted (1/20) in cold $HClO_4$ (final concentration 25 mM), filtered and injected immediately into the column (20 μL). Culture supernatants were used since it was shown that MDA is almost completely released to culture medium within 1 h after production [29]. Conjugated dienes were determined by analysis of the second derivate of the spectrum of cellular lipids measured between $\lambda = 220$–300 nm. Lipids from cell monolayers were extracted by the Folch method [9], the solvent was evaporated at 40 °C under $N_2$ stream and the lipids dissolved in cyclohexane before spectrophotometer measurement. These conjugated dienes present characteristic absorption peaks around 233 nm (*trans,trans* isomers) and 242 nm (*cis,trans* isomers) that become minima in the $d^2A/d\lambda^2$ of the spectrum [48].

*Characterization of the injury*

To evaluate the role of iron in the damage, cell monolayers were pre-incubated with culture medium supplemented with 10 mM DFO (Sigma D9533) for 30 min at 37 °C. Afterwards, they were washed with PBS and incubated in the corresponding condition (without additives, with Fe:8HQ or preserved at 0 °C) for 4 or 24 h. Then, viability by MTT reduction assay was measured.

In order to assess the contribution of hydroxyl radicals, the cells were incubated for 4 or 24 h in the selected condition together with 2.5 or 10% $Me_2SO$. After incubation, viability by MTT reduction assay was measured.

*Statistical analysis*

Groups of data were compared by analysis of variance (ANOVA) followed by standard parametric tests. The analyses were performed with InStat 3.05 (GraphPad Software Inc.) free software.

## Results

Hypothermia did not cause an observable damage when cells were incubated in mUW (Fig. 1). While control cultures (maintained at 37 °C in DMEM + FBS) continued to grow after 72 h incubation, cells submitted to 0 °C in mUW seemed to get into an arrested state as MTT reduction activities did not rise or decrease during this time (Fig. 1A). This was also noticed in LDH release results immediately after cold exposure (Fig. 1B) and following a period of rewarming (Fig. 1C). Once again, the elevated LDH leakage displayed by control cultures was due to detachment of overgrown cultures as confirmed by microscopic inspection and cold incubated cultures showed less than 5% LDH release even after 72 h exposure (Fig. 1B and C). In agreement with this, it was found mild o no increments in $O_2^{\cdot -}$ (Fig. 1D and Fig. S1) and MDA (Table 1) levels.

Afterward, incubation was tested at 0 °C in K–H, DMEM + FBS and HTK. As shown in Fig. 2A, incubation in hypothermia in K–H and DMEM + FBS was not adequate to this study because after 4 h of incubation, no damage was detected, and after 24 h the

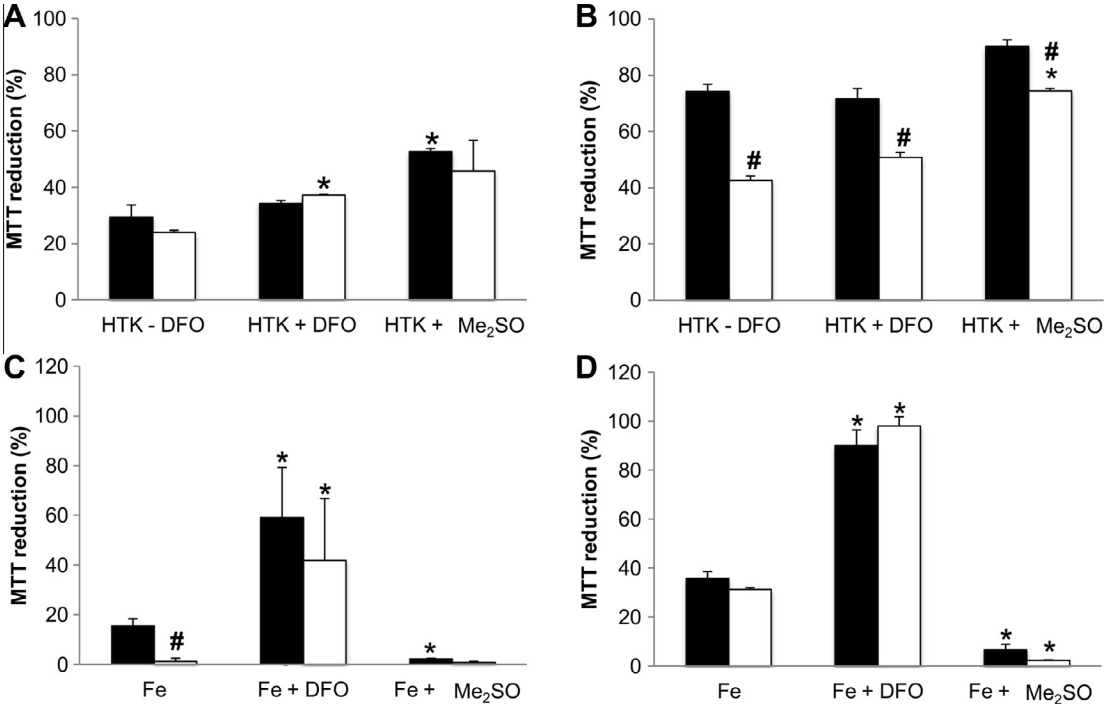


**Fig. 3.** Effects of addition of DFO and $Me_2SO$ on viability/growth after 24 h of incubation measured by MTT reduction assay. (A and C) Cells in exponential growth phase (seeded at $35 \times 10^3$ cells/cm$^2$) preserved at 0 °C in HTK solution and incubated in presence of 5 μM iron, respectively. (B and D) Arrested cells (seeded at $10^5$ cells/cm$^2$) preserved at 0 °C in HTK solution and incubated in presence of 5 μM iron, respectively. Black bars: Cos-7/pcDNA3 cells. White bars: Cos-7/pFNR cells, $n = 3$ experiments, in duplicate. $^*P < 0.05$, different from cells under control condition. $^{\#}P < 0.05$, different from Cos-7/pcDNA3 group.

totality of the cells was dead, precluding an analysis of the damage or a detection of differences between groups of cells. Instead, HTK incubated cells showed a different behavior; a good degree of damage (approximately 50%) was observed after 24 h at 0 °C as measured by MTT reduction levels (Fig. 2A). Damage of the two cell lines was confirmed by LDH release after rewarming (Fig. 2C). It was not found LDH leakage immediately after cold incubation (Fig. 2B), as it is usually seen for injuries produced by hypothermia that are only displayed after restoration of physiological conditions. Cos-7/pcDNA3 and Cos-7/pFNR, however, presented similar levels of damage and, as a consequence, no protection was exhibited by FNR for cells expressing this enzyme (Fig. 2B and C). MDA levels were much lower than those of Cos-7/pcDNA3 and Cos-7/pFNR maintained at 37 °C, which indicates that no oxidative damage had occurred to lipids (Table 1).

According to this result, it was analyzed whether the loss of viability was prevented by DFO and $Me_2SO$ (Fig. 3A and B). It was found a different degree of reversion of damage depending on the growing phase of the monolayer. When cells were at an exponential growing phase (Fig. 3A), differences in damage between cell lines (as in Fig. 2) were not observed and a modest amelioration of MTT reduction by DFO for Cos-7/pFNR and by $Me_2SO$ for Cos-7/pcDNA3 was detected. When monolayers were arrested for having reached confluency (Fig. 3B), Cos-7/pFNR cells resulted to be more susceptible to hypothermia exposure and the slight protection exerted by DFO for this line disappeared; both lines were now rescued by $Me_2SO$ treatment (Fig. 3B).

To ascertain the production of an oxidative damage mediated by iron in our system and to test FNR protection toward damage mediated by this cation, as proposed, it was decided to directly incubate the cultures with Fe:8HQ (selection of iron concentration on Fig. S2) (Fig. 4). It was observed a marked loss of viability as measured by MTT reduction and LDH release (Fig. 4A and B, respectively) with no significant rise in $O_2^{\cdot-}$ levels, as expected (Fig. 4C). It was found again more injury towards Cos-7/pFNR than for the control line Cos-7/pcDNA3 when accounting for LDH leakage and conjugated diene levels (Fig. 4B, Table 2), which was not observed for MTT reduction. In these experiments, DFO clearly protected from damage (Fig. 3C and D) as anticipated. Unfortunately, $Me_2SO$ resulted deleterious for cultures at 37 °C, even at low concentration (2.5%), impeding analysis of its effect (Fig. 3C and D). $OH^{\cdot-}$ did not rise significantly when measured in exponentially growing cultures (Table 3). Once more, regarding growing state of cells, it was observed a quantitatively different effect on exponential growing vs. stationary phase culture being the latter almost completely protected by DFO, differently from what was seen at 0 °C in HTK (compare Fig. 3B and D).

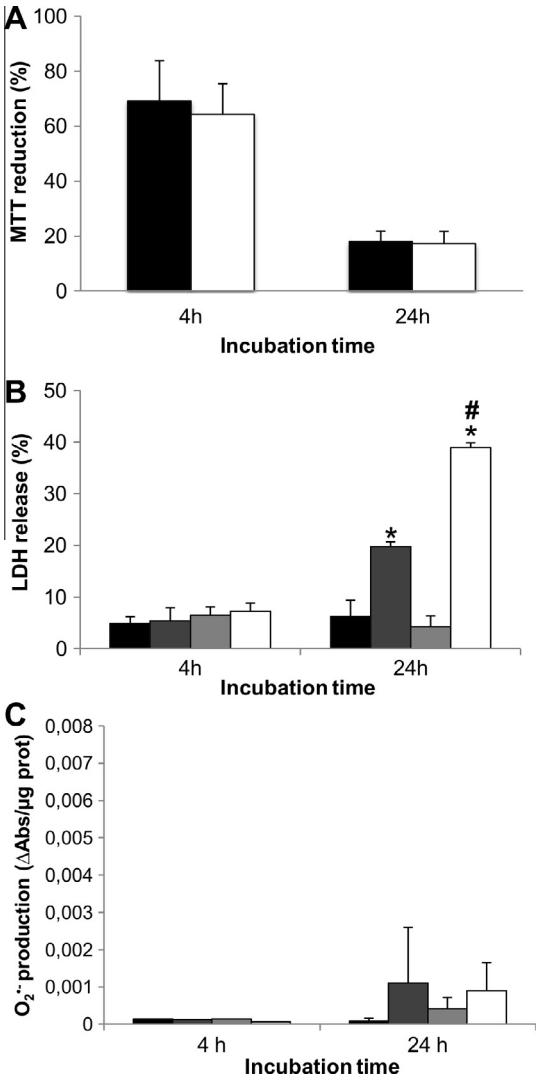


**Fig. 4.** Effects of 5 μM iron load. (A) Cell viability/growth measured by MTT reduction assay. The results were expressed in percentage respect to control cells incubated at 37 °C. (B) Cell damage after incubation measured by LDH release. (C) Superoxide anion production measured by NBT reduction assay. The results are expressed in absorbance units at 520 nm per μg of protein. Black and dark gray bars: Cos-7/pcDNA3 cells incubated at 37 °C without and with added iron, respectively. Light gray and white bars: Cos-7/pFNR cells incubated at 37 °C without and with added iron, respectively, $n = 3$ experiments, in duplicate. $^{*}P < 0.05$, different from the control condition. $^{\#}P < 0.05$, different from Cos-7/pcDNA3 group.

**Table 2**
Lipid peroxidation. Conjugated diene levels, expressed in AU corresponding to the minima near $\lambda = 233$ nm and $\lambda = 242$ nm in spectral scanning (200–300 nm).

| Incubation condition | $\lambda^2/dA^2$ minima | Cos-7/pcDNA3 (AU)[a] | Cos-7/pFNR (AU)[a] |
|---|---|---|---|
| 37 °C | $\lambda = 230.4$ nm | 0.283 ± 0.017 | 0.280 ± 0.015 |
| | $\lambda = 244.4$ nm | 0.090 ± 0.002 | 0.120 ± 0.014[b] |
| 5 μM Fe | $\lambda = 229.9$ nm | 0.440 ± 0.035 | 0.440 ± 0.028 |
| | $\lambda = 244.2$ nm | 0.117 ± 0.010 | 0.182 ± 0.016[b] |

[a] Results are expressed in arbitrary units (AU).
[b] Different from Cos-7/pcDNA3 in the same incubation condition; $n = 3$ experiments.

## Discussion

The temperature used in organ preservation for transplantation is given by the ice bath in which donor organs are transported and is usually near 0 °C. Some perfusion preservation techniques employ other temperatures – around 10 °C – obtaining better results but with a consequently reduced time window [54]. This difference can be explained when examining Arrhenius plots for biochemical reactions or metabolism rates such as oxygen consumption. These plots show linear relationships of viability vs. temperature with abrupt breaks that change the slope/rate of survival [21,22,50,54]. This indicates that the rate-limiting step had changed from one side of the break to the other as temperature decreases, which is not unexpected in complex systems such as cells or tissues [21,22,50,54]. The temperature at which the break is observed varies among cells originated from different organs and species [21,22,50]. Therefore, to slow down biochemical processes and to prolong preservation time, it would be desirable to lower preservation temperatures to near 0 °C; however, conditions should be improved since this is also more detrimental to the tissue being stored.

In this work we investigated the capacity of *P. sativum* FNR to protect Cos-7 cells from injury induced by exposure to hypothermia at 0 °C.

In order to preserve organs and cells from the aforementioned injury, a variety of solutions have been developed [3]. These are intracellular type solutions: generally hypertonic solutions, with a composition designed to restrict the passive exchange of water and ions during the cold exposure when membrane ion pumps are inhibited. This is achieved by raising the concentration of potassium and diminishing the concentration of sodium in order to imitate the intracellular space. These solutions usually include a non-permeable anion (lactobionate or gluconate) that partially replace chloride anion and provide an osmotic support to balance the oncotic pressure generated by the macromolecules associated to counter ions of the intracellular space [50]. On the other hand, extracellular type solutions are, in general, poor preservation solutions because they do not counteract the passive biophysical processes that trigger the mentioned phenomenon [50].

We first submitted cells to cold incubation in mUW, due to the fact that other authors observed a marked damage after few hours [34,42,44]. We saw almost no loss of viability or augmentation of lipid peroxidation even after 72 h of incubation. Several facts could have caused this lack of effect. First, Cos-7 cells are adapted to culture conditions that are more oxidative than the tissue of origin, as it is known to be the case for many established cell lines – for example, oxygen tension in the culture medium inside $CO_2$ incubators is higher. Second, mUW (Table S1) has been modified in our laboratory in order to make it more protective than original UW (or Viaspan®) for isolated cells, the latter being intended for whole organ preservation. In particular, hydroxyethyl starch was replaced by polyethylene glycol (PEG), which increases the osmolarity of this solution avoiding swelling and is also proposed to protect isolated cells by interacting with the cellular membrane and coating it [5,6,25]. As well, the unstable component glutathione is added in its reduced form immediately before use, assuring in this way its reducing capacity during storage [47,52].

We found that HTK (or Celsior®) solution used in its original formulation resulted less protective in our conditions. Nonetheless, Cos-7/pFNR cells were not more resistant to hypothermia than the control line that does not express FNR. Moreover, they appear to suffer more serious damage when this was measured by LDH release (Fig. 2C) and by MTT at a quiescent state (Fig. 3B). The damage was only slightly reversed by DFO and $Me_2SO$ when cells were growing at exponential rate but strongly reversed by $Me_2SO$ when in arrested state, which indicates an almost exclusive participation of $OH^{\cdot -}$ under this last condition (Fig. 3A and B). It seems that under exponential growth, damage provoked by hypothermia has a mixed nature with an iron-independent component – which seems to be more extensive – and an iron-dependent component – as already reported by Rauen et al. [42]. This could be related to an $O_2^{\cdot -}$ increase – not measured for HTK but found to be present in cells incubated in mUW (Fig. S1) – since, although the magnitude of the damage is very different, we did not expect that the nature of it would be dissimilar. On the contrary, under arrested state, cells displayed a damage that seems to be only of one nature, involving $OH^{\cdot -}$ solely, and apparently not derived from iron increase as DFO did not protected Cos-7 cells (Fig. 3B). The fact that the extension of the damage is smaller in arrested monolayers than in exponentially growing monolayers supports the idea of the disappearance of one of the injury components.

From the results presented, we cannot demonstrate that FNR is protective against an iron-dependent hypothermic injury and so, we studied FNR effect under an insult directly provoked by iron load. Again, we observed that FNR was not protective and even enhanced the injury (Fig. 4B and Table 3). DFO, as expected, reversed the damage (Fig. 3C and D) to a different extent to what observed for HTK incubation.

It is very probable that the diaphorase activity of overexpressed FNR causes the exhaustion of NADPH pools as it is used as an electron donor [8,24]. This nucleotide provides reduction equivalents for regeneration of oxidized antioxidants, such as GSSG. Consequently, the reduced metabolic rate in arrested cells impedes repletion of NADP(H) possibly exacerbating the damage as already seen in *E. coli* [20] and in our previous work on Cos-7 cells exposed to MV [27]. Furthermore, the injury due to hypothermia in endothelial hepatic cells that is an exclusively iron-related damage has been demonstrated to be an energy-dependent process [40,41]. This evidence is congruent with our results showing that iron-dependent component of the injury in confluent cells has disappeared or was greatly diminished as no reversion by DFO was observed (Fig. 3B).

In general, we found that parameters measuring oxidative stress damage, as MDA, conjugated dienes, $O_2^{\cdot -}$ and $OH^{\cdot -}$ levels did not rise with any of the treatments applied even though viability decreased. We have previously worked with primary cells that present some disadvantages such as the need of isolating cells for every experiment, with the consequent usage of a great number of laboratory animals and its associated variability, very low transfection efficiencies with non-viral vectors, etc. Working with an established cell line obviously avoids these inconveniencies but, in our experiments, Cos-7 cells seem to present an elevated resistance to oxidative insults. This is not surprising because they are adapted to culture environment that is more oxidative than the tissue of origin, as already mentioned above.

When we integrated the present results with those previously obtained by our group [27,28], we could observe that the overexpression of FNR is beneficial in some situations – such as implantation of preserved primary hepatocytes and exposure of Cos-7 cells to $H_2O_2$ – and not protective or even unfavorable in other conditions – such as exposure of Cos-7 cells to MV, hypothermia and iron overload. As FNR was demonstrated to be clearly protective in bacterial, cyanobacterial and plant models [19,20,31,35,53] then, it is necessary to fully characterize the mechanisms employed by this enzyme in mammalian cells in order to fine-tune its activity – by choosing cellular type, growth cycle phase, level

**Table 3**
Hydroxyl radical levels measured by deoxy-ribose method as MDA equivalents produced.

| Incubation condition[a] | Cos-7/pcDNA3(Abs 532 nm) | Cos-7/pFNR (Abs 532 nm) | Cos-7/pcDNA3 (ΔAbs)[b] | Cos-7/pFNR (ΔAbs)[b] | Cos-7/pcDNA3 (μM) | Cos-7/ pFNR(μM) |
|---|---|---|---|---|---|---|
| 37 °C (−DR) | 0.000 ± 0.000 | 0.037 ± 0.001 | 0.034 ± 0.001 | 0.039 ± 0.001 | 5.77 ± 0.21 | 6.81 ± 0.27 |
| 37 °C (+DR) | 0.034 ± 0.003 | 0.076 ± 0.004 | | | | |
| 6.56 μM Fe (−DR) | 0.022 ± 0.002 | 0.058 ± 0.007 | 0.027 ± 0.002 | 0.018 ± 0.000 | 4.31 ± 0.42 | 2.43 ± 0.09[c] |
| 6.56 μM Fe (+DR) | 0.049 ± 0.005 | 0.076 ± 0.003 | | | | |

[a] Reaction mixture with (+) or without (−) added deoxyribose (DR).
[b] ΔAbs: difference of absorbances read in the presence (+) and absence (−) of DR.
[c] Different from Cos-7/pcDNA3 in the same incubation condition; $n = 3$ experiments.

and intracellular site of expression [1,8]. In this way, we would be able to determine whether FNR is applicable or not to the problem of oxidative stress encountered in a wide variety of situations such as organ preservation as mentioned here or in many diseases as cancer or Alzheimer among others [4,12,51].

## Conflict of interest

No conflict of interests are declared for this work.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cryobiol.2013.05.005.

## References

[1] P.G. Arthur, X. Niu, P. Rigby, J.H. Steer, G.P. Jeffrey, Oxidative stress causes a decline in lysosomal integrity during hypothermic incubation of rat hepatocytes, Free Radic. Biol. Med. 44 (2008) 24–33.
[2] C. Auclair, M. Torres, J. Hakim, Superoxide anion involvement in NBT reduction catalyzed by NADPH-cytochrome P-450 reductase: a pitfall, FEBS Lett. 89 (1978) 26–28.
[3] F.O. Belzer, J.H. Southard, Principles of solid-organ preservation by cold storage, Transplantation 45 (1988) 673–676.
[4] R.J. Castellani, P.I. Moreira, G. Liu, J. Dobson, G. Perry, M.A. Smith, X. Zhu, Iron: the redox-active center of oxidative stress in Alzheimer disease, Neurochem. Res. 32 (2007) 1640–1645.
[5] D. Dutheil, I. Rioja-Pastor, C. Tallineau, J.M. Goujon, T. Hauet, G. Mauco, I. Petit-Paris, Protective effect of PEG 35,000 Da on renal cells: paradoxical activation of JNK signaling pathway during cold storage, Am. J. Transplant. 6 (2006) 1529–1540.
[6] D. Dutheil, G.A. Underhaug, I. Petit-Paris, G. Mauco, H. Holmsen, Polyethylene glycols interact with membrane glycerophospholipids: is this part of their mechanism for hypothermic graft protection?, J Chem. Biol. 2 (2009) 39–49.
[7] M. Duval, C. Plin, A. Elimadi, D. Vallerand, J.P. Tillement, D. Morin, P.S. Haddad, Implication of mitochondrial dysfunction and cell death in cold preservation-warm reperfusion-induced hepatocyte injury, Can. J. Physiol. Pharmacol. 84 (2006) 547–554.
[8] O. Elroy-Stein, Y. Bernstein, Y. Groner, Overproduction of human Cu/Zn-superoxide dismutase in transfected cells: extenuation of paraquat-mediated cytotoxicity and enhancement of lipid peroxidation, EMBO J. 5 (1986) 615–622.
[9] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[10] R.I. Freshney, Culture of Animal Cells: A Manual of Basic Technique, 5th ed., Wiley-Liss, New York, 2005.
[11] B.J. Fuller, J. Lunec, G. Healing, S. Simpkin, C.J. Green, Reduction of susceptibility to lipid peroxidation by desferrioxamine in rabbit kidneys subjected to 24-hour cold ischemia and reperfusion, Transplantation 43 (1987) 604–606.
[12] D. Galaris, V. Skiada, A. Barbouti, Redox signaling and cancer: the role of "labile" iron, Cancer Lett. 266 (2008) 21–29.
[13] J.M. Gutteridge, Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates, FEBS Lett. 128 (1981) 343–346.
[14] J.M. Gutteridge, B. Halliwell, The deoxyribose assay: an assay both for 'free' hydroxyl radical and for site-specific hydroxyl radical production, Biochem. J. 253 (1988) 932–933.
[15] G. Healing, J. Gower, B. Fuller, C. Green, Intracellular iron redistribution. An important determinant of reperfusion damage to rabbit kidneys, Biochem. Pharmacol. 39 (1990) 1239–1245.
[16] S.K. Jonas, P.A. Riley, The effect of ligands on the uptake of iron by cells in culture, Cell Biochem. Funct. 9 (1991) 245–253.
[17] O. Kakhlon, Z.I. Cabantchik, The labile iron pool: characterization, measurement, and participation in cellular processes(1), Free Radic. Biol. Med. 33 (2002) 1037–1046.
[18] F. Karatas, M. Karatepe, A. Baysar, Determination of free malondialdehyde in human serum by high-performance liquid chromatography, Anal. Biochem. 311 (2002) 76–79.
[19] A.R. Krapp, N. Carrillo, Functional complementation of the mvrA mutation of Escherichia coli by plant ferredoxin-NADP+ oxidoreductase, Arch. Biochem. Biophys. 317 (1995) 215–221.
[20] A.R. Krapp, V.B. Tognetti, N. Carrillo, A. Acevedo, The role of ferredoxin-NADP+ reductase in the concerted cell defense against oxidative damage – studies using Escherichia coli mutants and cloned plant genes, Eur. J. Biochem. 249 (1997) 556–563.
[21] J. Kruuv, D.J. Glofcheski, J.R. Lepock, Evidence for two modes of hypothermia damage in five cell lines, Cryobiology 32 (1995) 182–190.
[22] J. Kruuv, J.R. Lepock, Factors influencing survival of mammalian cells exposed to hypothermia VI. Effects of prehypothermic hypoxia followed by aerobic or hypoxic storage at various hypothermic temperatures, Cryobiology 32 (1995) 191–198.
[23] C. Legrand, J.M. Bour, C. Jacob, J. Capiaumont, A. Martial, A. Marc, M. Wudtke, G. Kretzmer, C. Demangel, D. Duval, Lactate dehydrogenase (LDH) activity of the cultured eukaryotic cells as marker of the number of dead cells in the medium [corrected], J. Biotechnol. 25 (1992) 231–243.
[24] S.I. Liochev, A. Hausladen, W.F. Beyer Jr., I. Fridovich, NADPH: ferredoxin oxidoreductase acts as a paraquat diaphorase and is a member of the soxRS regulon, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 1328–1331.
[25] J.E. Mack, J.A. Kerr, P.K. Vreugdenhil, F.O. Belzer, J.H. Southard, Effect of polyethylene glycol on lipid peroxidation in cold-stored rat hepatocytes, Cryobiology 28 (1991) 1–7.
[26] F. Magni, G. Panduri, N. Paolocci, Hypothermia triggers iron-dependent lipoperoxidative damage in the isolated rat heart, Free Radic. Biol. Med. 16 (1994) 465–476.
[27] M.G. Mediavilla, G.A. Di Venanzio, E.E. Guibert, C. Tiribelli, Heterologous ferredoxin reductase and flavodoxin protect Cos-7 cells from oxidative stress, PLoS One 5 (2010) e13501.
[28] M.G. Mediavilla, A. Krapp, N. Carrillo, J.V. Rodriguez, C. Tiribelli, E.E. Guibert, Efficient cold transfection of pea ferredoxin-NADP(H) oxidoreductase into rat hepatocytes, J. Gene Med. 8 (2006) 306–313.
[29] I. Morel, G. Lescoat, J. Cillard, N. Pasdeloup, P. Brissot, P. Cillard, Kinetic evaluation of free malondialdehyde and enzyme leakage as indices of iron damage in rat hepatocyte cultures. Involvement of free radicals, Biochem. Pharmacol. 39 (1990) 1647–1655.
[30] X. Niu, P.G. Arthur, G.P. Jeffrey, Iron and oxidative stress in cold-initiated necrotic death of rat hepatocyte, Transplant. Proc. 42 (2010) 1563–1568.
[31] J.F. Palatnik, V.B. Tognetti, H.O. Poli, R.E. Rodriguez, N. Blanco, M. Gattuso, M.R. Hajirezaei, U. Sonnewald, E.M. Valle, N. Carrillo, Transgenic tobacco plants expressing antisense ferredoxin-NADP(H) reductase transcripts display increased susceptibility to photo-oxidative damage, Plant J. 35 (2003) 332–341.
[32] S.M. Peters, U. Rauen, M.J. Tijsen, R.J. Bindels, C.H. van Os, H. de Groot, J.F. Wetzels, Cold preservation of isolated rabbit proximal tubules induces radical-mediated cell injury, Transplantation 65 (1998) 625–632.
[33] F. Petrat, H. de Groot, R. Sustmann, U. Rauen, The chelatable iron pool in living cells: a methodically defined quantity, Biol. Chem. 383 (2002) 489–502.
[34] G. Pless, I.M. Sauer, U. Rauen, Improvement of the cold storage of isolated human hepatocytes, Cell Transplant. 21 (2012) 23–37.
[35] P.J. Pomposiello, B. Demple, Identification of SoxS-regulated genes in Salmonella enterica serovar typhimurium, J. Bacteriol. 182 (2000) 23–29.
[36] S.Y. Qian, G.R. Buettner, Iron and dioxygen chemistry is an important route to initiation of biological free radical oxidations: an electron paramagnetic resonance spin trapping study, Free Radic. Biol. Med. 26 (1999) 1447–1456.
[37] U. Rauen, H. de Groot, Cold-induced release of reactive oxygen species as a decisive mediator of hypothermia injury to cultured liver cells, Free Radic. Biol. Med. 24 (1998) 1316–1323.
[38] U. Rauen, B. Elling, H. de Groot, Injury to cultured liver endothelial cells after cold preservation: mediation by reactive oxygen species that are released independently of the known trigger hypoxia/reoxygenation, Free Radic. Biol. Med. 23 (1997) 392–400.
[39] U. Rauen, B. Elling, E.R. Gizewski, H.G. Korth, R. Sustmann, H. de Groot, Involvement of reactive oxygen species in the preservation injury to cultured liver endothelial cells, Free Radic. Biol. Med. 22 (1997) 17–24.
[40] U. Rauen, M. Hanssen, W. Lauchart, H.D. Becker, H. de Groot, Energy-dependent injury to cultured sinusoidal endothelial cells of the rat liver in UW solution, Transplantation 55 (1993) 469–473.
[41] U. Rauen, K. Hintz, M. Hanssen, W. Lauchart, H.D. Becker, H. de Groot, Injury to cultured liver endothelial cells during cold preservation: energy-dependent versus energy-deficiency injury, Transpl. Int. 6 (1993) 218–222.
[42] U. Rauen, U. Kerkweg, H. de Groot, Iron-dependent vs. iron-independent cold-induced injury to cultured rat hepatocytes: a comparative study in physiological media and organ preservation solutions, Cryobiology 54 (2007) 77–86.
[43] U. Rauen, U. Kerkweg, D. Weisheit, F. Petrat, R. Sustmann, H. de Groot, Cold-induced apoptosis of hepatocytes: mitochondrial permeability transition triggered by nonmitochondrial chelatable iron, Free Radic. Biol. Med. 35 (2003) 1664–1678.
[44] U. Rauen, F. Petrat, T. Li, H. de Groot, Hypothermia injury/cold-induced apoptosis–evidence of an increase in chelatable iron causing oxidative injury in spite of low O2-/H2O2 formation, FASEB J. 14 (2000) 1953–1964.
[45] U. Rauen, B. Polzar, H. Stephan, H.G. Mannherz, H. de Groot, Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species, FASEB J. 13 (1999) 155–168.

[46] U. Rauen, I. Reuters, A. Fuchs, H. de Groot, Oxygen-free radical-mediated injury to cultured rat hepatocytes during cold incubation in preservation solutions, Hepatology 26 (1997) 351–357.
[47] J.V. Rodriguez, M.E. Mamprin, M.G. Mediavilla, E.E. Guibert, Glutathione movements during cold preservation of rat hepatocytes, Cryobiology 36 (1998) 236–244.
[48] O. Sergent, I. Morel, P. Cogrel, M. Chevanne, N. Pasdeloup, P. Brissot, G. Lescoat, P. Cillard, J. Cillard, Simultaneous measurements of conjugated dienes and free malondialdehyde, used as a micromethod for the evaluation of lipid peroxidation in rat hepatocyte cultures, Chem. Phys. Lipids 65 (1993) 133–139.
[49] M.J. Steinbeck, A.U. Khan, W.H. Appel Jr., M.J. Karnovsky, The DAB-Mn++ cytochemical method revisited: validation of specificity for superoxide, J. Histochem. Cytochem. 41 (1993) 1659–1667.
[50] M.J. Taylor, Biology of Cell Survival in the ColdThe Basis for Biopreservation of Tissues and Organs. Advances in Biopreservation., CRC Press, Boca Raton, 2006, pp. 15–62.
[51] C. Thomas, M.M. Mackey, A.A. Diaz, D.P. Cox, Hydroxyl radical is produced via the Fenton reaction in submitochondrial particles under oxidative stress: implications for diseases associated with iron accumulation, Redox. Rep. 14 (2009) 102–108.
[52] M. Vairetti, P. Griffini, G. Pietrocola, P. Richelmi, I. Freitas, Cold-induced apoptosis in isolated rat hepatocytes: protective role of glutathione, Free Radic. Biol. Med. 31 (2001) 954–961.
[53] N. Yousef, E.K. Pistorius, K.P. Michel, Comparative analysis of idiA and isiA transcription under iron starvation and oxidative stress in Synechococcus elongatus PCC 7942 wild-type and selected mutants, Arch. Microbiol. 180 (2003) 471–483.
[54] M.A. Zieger, D.J. Glofcheski, J.R. Lepock, J. Kruuv, Factors influencing survival of mammalian cells exposed to hypothermia IV. Effects of iron chelation, Cryobiology 27 (1990) 452–464.