Hypothermic Injury: the Mitochondrial Calcium, ATP and ROS Love-Hate Triangle out of Balance

Paul-Thomas Brinkkoetter¹,*, Hui Song¹,4,*, Ralf Lösel²,*, Ulf Schnetzke¹,*, Uwe Gottmann¹, Yuxi Feng¹, Christine Hanusch³, Grietje C. Beck³, Peter Schnuelle¹, Martin Wehling², Fokko J. van der Woude¹ and Benito A. Yard¹

¹Vth Medical Department, ²Department of Clinical Pharmacology and ³Department of Anesthesiology, University Hospital of Mannheim, University of Heidelberg, Mannheim, ⁴School of Dentistry, Shandong University, Jinan, *P.T.B., H. S., R.L., and U.S. contributed equally to this work

Key Words
Hypothermia • Endothelial cell • Catecholamine • Mitochondria

Abstract
Background/Aims: Catecholamines prevent hypothermic cell death which accounts for severe tissue damage and impaired allograft function after prolonged organ preservation. Here, we identified cellular processes which govern hypothermia-mediated cell death in endothelial cells and how they are influenced by dopamine. Methods: Lactate dehydrogenase assay, intracellular ATP, reactive oxygen species and reduced thio-group measurement, intracellular calcium measurement and mitochondrial calcium staining were performed in the study. Results: Intracellular ATP was almost completely depleted within 12 hrs of hypothermic preservation in untreated human umbilical vein endothelial cells (HUVEC), while dopamine pre-treatment significantly delayed ATP depletion. 4 hrs after hypothermia a redox imbalance was observed in untreated cells, which increased with the duration of hypothermia. The redox imbalance was primarily caused by depletion of SH reduction equivalents and was significantly inhibited by dopamine. In addition, hypothermia-induced Ca²⁺ influx and mitochondrial Ca²⁺ accumulation were both prevented by dopamine. The protective effect of dopamine was abrogated by ionomycin and sodium azide and partly by oligomycin and CCCP. Conclusions: Our data demonstrated that loss of intracellular ATP, generation of a redox imbalance and accumulation of intracellular Ca²⁺ underlie cold preservation injury. Dopamine improves the redox balance, prevents intracellular Ca²⁺ accumulation and delays ATP depletion.

Introduction
Mitochondria are the major sites of ATP synthesis in eukaryotes. ATP is generated via oxidative phosphorylation (ox-phos), in which electrons are liberated from reduced substrates and finally delivered to O₂. Approximately 2% of consumed oxygen is converted into reactive oxygen species (ROS). During ox-phos, the respiratory chain complexes (complex I-IV) establish an H⁺ gradient, which is then utilized to drive ATP synthesis via complex V, i.e. ATP synthase. ROS production ensues from the activity of the respiratory chain. The superoxide anion is produced at two major sites in...
the respiratory chain, i.e. in the ubiquinone- (Q) cycle via the ubisemiquinone radical intermediate [1, 2] and in complex I through a backward electron transfer from succinate to NAD+ [3]. Manganese superoxide dismutase (MnSOD) converts superoxide into hydrogenperoxide which, in turn, is reduced to hydroxyl radicals by Fenton chemistry [4]. Apart from their role in ATP synthesis, mitochondria also play a pivotal role in other cellular processes such as calcium (Ca2+) homeostasis [5, 6].

Under physiological conditions mitochondrial Ca2+, ATP synthesis and ROS production are tightly regulated in a discrete balance, designated as the love-hate triangle [7]. Ca2+ is a physiological stimulator of ox-phos to balance mitochondrial ATP output and cellular ATP demand [8-10]. However, when mitochondrial Ca2+ overload occurs, Ca2+ becomes patho-physiologically relevant and is embodied in the regulation of opening of the mitochondrial permeability transition (MPT) pore. Opening of the MPT pore is mediated by high mitochondrial Ca2+ concentrations [Ca2+]m and is facilitated by ROS. The mitochondrial membrane potential completely dissipates at a high open probability (Po) of the MPT pore. Unstable membrane potential and redox transitions can occur under a variety of pathological conditions, e.g. ischemia/reperfusion injury, and might have negative consequences for mitochondrial integrity and cellular survival [11, 12].

Hypothermia and ischemia are two major hurdles in hypothermic organ preservation as they negatively affect mitochondrial function. Although it has been acknowledged that prolonged hypothermic preservation of organ allografts is detrimental to organ viability [13], the cellular events leading to hypothermic injury have not been elucidated completely. Nevertheless, various studies have highlighted the importance of ROS production, impairment of Ca2+ homeostasis and ATP depletion in hypothermic injury [14, 15].

In order to handle cold ischemic conditions, hibernating mammals have developed a number of strategies to limit the problems of Ca2+ homeostasis, ATP synthesis and ROS production. A decreased ox-phos [16], the expression of uncoupling proteins in brown adipose tissue [17], as well as an increased Ca2+ uptake in the sarco/endoplasmic reticulum [18, 19] are amongst mechanisms used by hibernating mammals to adapt to long periods of hypothermia and reduced oxygen pressure.

We and others have demonstrated that dihydroxy-phenolic compounds [20-22] and iron chelators [23, 24] can protect endothelial- and tubular epithelial cells against hypothermia mediated cell death. In the present study we hypothesized that these compounds mediate protection by balancing the mitochondrial Ca2+- ATP - ROS love-hate triangle during hypothermia.

Materials and Methods

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as described previously [25]. The cells were cultured in endothelial cell growth medium (EGM) (Promocell, Heidelberg, Germany) in T25 flasks (Greiner, Frickenhausen, Germany) coated with gelatine (1%). Confluent monolayers were passaged by Trypsin/EDTA (Sigma-Aldrich, St. Louis, MO). Characterization of endothelial cells was performed on the basis of a positive uptake of acetylated LDL, Factor VIII related antigen and PECAM (CD31) expression, and a negative staining for alpha smooth muscle actin. All experiments were carried out at 100% confluence.

Dopamine treatment

HUVEC were incubated for 2hrs with 25µM of dopamine (DA, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany). The culture flasks were washed 3 times with 1ml of PBS (Invitrogen, Karlsruhe, Germany) and stored for 24hrs at 4°C in HTK-, UW-solution or phenol-red free medium (Promocell). Since there was no difference between the use of the preservation solutions or phenol red free medium with respect to cell damage after hypothermic preservation, in all experiments described in this paper, the cells were stored in phenol-red free medium. In some experiments EDTA, BAPTA-AM, thapsigargin, myothioziale, Na-azide, oligomycin, ionomycin, CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (all from Sigma-Aldrich, St. Louis, MO) were added to the medium during hypothermia.

LDH assay

Lactate dehydrogenase assays were performed as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany). Briefly, HUVEC were plated in 24-well plates, grown until confluence and stimulated with 25µM dopamine (DA) for 2hrs. The plates were washed 3 times with 1ml of PBS and stored for 24hrs at 4°C in phenol-red free medium. A 100µl aliquot of each supernatant was used to determine LDH release. In each experiment 100µl of phenol-red free medium was used as blank. The results are expressed as OD490nm, corrected for the blank value.

Measurement of reactive oxygen species

The intracellular formation of reactive oxygen species (ROS) was detected using the fluorescent probe CM-H2-DCFDA (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions. The formation of superoxide anions and hydroxyl radicals can both be assessed by this method. HUVEC grown in 96-well plates were pre-treated with 25µM DA for 2hrs. Then the cells were loaded with 10µM of CM-H2-DCFDA for 45min at 37°C. The cells were incubated at 37°C and 4°C respectively and analysed by serial measurements in a Spectra

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Fluor Plus Fluorescent 96-well plate reader (Tecan Deutschland, Crailsdorf, Germany).

Assessment of SH reduction equivalents
Intracellular “-SH groups” were quantified with a standard assay based on the reduction of 5,5’- Dithiobis-2-Nitrobenzoic acid (DTNB, Sigma). Cells were lysed in 10mM Tris-buffer w/ 1% Triton X-100 (Sigma), followed by centrifugation at 15,000rpm for 10 minutes. The supernatant was incubated with phosphate buffer containing DTNB at room temperature for 60 minutes. The quantification was conducted at 412nm using a spectrophotometer (Spectra Fluor Plus 96-well plate reader, Tecan).

Assessment of intracellular ATP
Confluent HUVEC monolayers were treated for 2hrs with 25µM dopamine or left untreated. Subsequently the cells were washed and stored at 4°C. Intracellular ATP was extracted at serial time points and measured by luciferase driven bioluminescence using the ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics, Mannheim, Germany).

Confocal microscopy for mitochondrial calcium staining
Reduced Rhod-2 AM (Invitrogen, Karlsruhe, Germany) was used to detect mitochondrial calcium levels. Rhod-2 AM stock solution dissolved in DMSO (Sigma) was incubated with Na-borohydride (NaBH₄, Sigma) solution for 10 minutes at room temperature. HUVECs cultured on coverslips were incubated with phenol-red free medium containing 5µM dihydroRhod-2 AM at 37°C for 40 minutes. The cells were washed with PBY twice and subjected to 4°C. In some cases cells were simultaneously co-incubated with MitoTracker Green (200nM, Invitrogen, Karlsruhe, Germany). Fluorescence images were obtained by confocal microscopy (Leica TCS SP2, Leica Microsystems, Heidelberg, Germany) under corresponding excitation and emission wave lengths. Quantification was performed by image analysis (Leica confocal software) and the results were expressed as relative levels of fluorescence intensity.

Measurements of intracellular Ca²⁺ concentrations
Measurements of intracellular Ca²⁺ concentrations were performed according to Koppel et al. [26]. HUVEC were incubated with phenol-red free medium containing 4µm Fura-2 AM (Invitrogen, Karlsruhe, Germany) at 37°C for 1hr. The cells were washed with PBS twice and treated with dopamine for 2hrs. After washing the cells with PBS twice, phenol-red free media was added and the cells were subjected to 4°C for 3hrs. Coverslips were then mounted into a thermostatically regulated microscope chamber. A Zeiss Axiovert 35 (Zeiss, Hanau, Germany) inverted fluorescence microscope, equipped with a fluorescence imaging camera (General Scanning, Planegg, Germany), was employed to detect fluorescence changes. Dual wavelength excitation at 340 and 380 nm was performed by an imaging system (Till Photonics, Planegg, Germany). After calibration, the following equation was used to relate the intensity ratios to cytosolic calcium levels: cytosolic calcium=Kd Q(R-Rmin)/(Rmax-R).

Statistical analysis
For statistical analysis unpaired Student’s t-test (Stats Direct 2.2.2) was applied when appropriate. A P-value of less than 0.05 was considered to be significant.

Results

Influence of hypothermia on intracellular ATP concentrations
Human umbilical vein endothelial cells (HUVEC) are extremely susceptible to hypothermic injury. Nevertheless, resistance can be acquired by appropriate pre-treatment using dopamine or related compounds, as evidenced by an inhibition of LDH release. In dopamine pre-treated HUVEC, protection against hypothermic injury was transient, lasting for approximately 48hrs (Fig. 1A).

To gain further insights into why dopamine pre-treated HUVEC are protected against hypothermic injury, we investigated changes in intracellular ATP levels during hypothermia. In HUVEC, 90% of intracellular ATP was rapidly lost during the first 12hrs. Although dopamine pre-treatment resulted in a significant retardation of ATP depletion, ATP levels still decreased after 24hrs of hypothermia (Fig. 1B).

Influence of hypothermia on intracellular redox balance
We next assessed whether hypothermia is affecting the intracellular redox balance. At early time points after hypothermia, ROS production was not significantly different from control cells kept at 37°C. Four hours after initiation of hypothermia however, the amount of SH-reduction equivalents was significantly decreased (Fig. 2A). As hypothermia continued, ROS production decreased approximately by 50% while the amount of SH-reduction equivalents dropped to less than 10% after 24hrs (Fig. 2B). Similarly, in dopamine pre-treated HUVEC, ROS production was decreased during hypothermia, but the loss of SH-reduction equivalents was significantly lower in these cells (Fig. 2A and B). To assess to what extent loss of SH-reduction equivalents and ROS production affects the redox balance, we calculated the ROS/SH ratio. In untreated versus dopamine treated HUVEC, the ROS/SH ratio was 2- and 7-fold higher, after 4 and 24hrs of hypothermia respectively (Fig. 2C).
Role of Ca\textsuperscript{2+} in hypothermia mediated cell death

The loss of ATP and the redox imbalance during hypothermia can affect calcium homeostasis by impairing ATP dependent ion channels. We therefore tested if a calcium influx occurs during hypothermia and if this was required for cell death. Our results showed that the

Fig 1. A: Dopamine pre-treatment renders HUVEC transiently resistant to hypothermia induced cell death. Untreated HUVEC (black circles) and dopamine treated (25µM for 2hrs) HUVEC (open circles) were subjected to hypothermia for different time periods. Directly after cold storage cell viability was assessed by LDH release in supernatants. All experimental conditions were performed in triplicate. The results of a representative experiment are expressed as mean LDH release ± SD. A total of 4 experiments were performed. B: Loss of intracellular ATP during hypothermia in HUVEC. Untreated HUVEC (black circles) and dopamine pre-treated (25µM for 2hrs) HUVEC (open circles) were subjected to hypothermia as in A. ATP concentrations were assessed before and directly after various time points of hypothermia. All experimental conditions were performed in triplicate. The results are expressed as mean % of ATP ± SD relative to the amount of ATP before hypothermia. A total of 5 experiments were performed.

Fig 2. Influence of hypothermia on intracellular redox balance of HUVEC. The amount of free SH-reduction equivalents and the production of ROS were assessed in untreated HUVEC (black bars) and dopamine pre-treated (25µM for 2hrs) HUVEC (grey bars) 4hrs (A) or 24hrs (B) after initiation of hypothermia. SH-reduction equivalents and ROS produced in HUVEC kept at 37°C for 4hrs (in Figure 2A) or for 24hrs (in Figure 2B) (open bars) was taken as 100 %. In A and B, the results are expressed as mean % of free SH-reduction equivalents or % of ROS ± SD relative to cells kept at 37°C. All experimental conditions were performed in triplicate, a total of 5 experiments were performed. C: The ratio of ROS and free SH-reduction equivalents after 4 and 24hrs of hypothermia. The results are expressed as mean ROS/SH ratio ± SD for all 5 experiments (*: P<0.01, unpaired t-test).
addition of EDTA to the preservation solution completely prevented hypothermia mediated cell death in HUVEC (Fig 3A). In time-course experiments, the addition of EDTA could prevent hypothermia mediated cell death when administered for up to 4hrs after initiation of hypothermia. Beyond this time point, addition of EDTA did not protect HUVEC from cell death. Similarly, when dopamine was added at different time points after hypothermia it was not protective when the cells were already subjected to hypothermia for more than 2hrs (Fig 3B).

Intracellular [Ca^{2+}], measured after the first 3hrs of hypothermia, was significantly higher in untreated than in dopamine treated HUVEC. When EDTA was added to the preservation solution no increase in intracellular [Ca^{2+}] occurred (Fig 3C). While addition of the calcium ionophore ionomycin completely abolished the protective effect of dopamine, this was not observed in the presence of EDTA. Similarly, addition of thapsigargin to release Ca^{2+} from intracellular stores, only affected dopamine mediated protection but not that of EDTA (Fig 3D). The intracellular Ca^{2+} chelator BAPTA-AM was also
protective against hypothermia mediated cell death.

To investigate if an increased Ca²⁺ influx resulted in mitochondrial Ca²⁺ accumulation, the mitochondrial calcium indicator Rhod-2 was used to assess changes in [Ca²⁺]ₘ. The specificity of Rhod-2 for mitochondrial calcium was demonstrated by double labelling with Mitotracker green and the absence of Rhod-2 staining when the cells were treated with CCCP to dissipate the mitochondrial membrane potential. Based on the time-course experiments with EDTA, we anticipated that calcium entry would occur within the first 4hrs of hypothermia. 3hrs of hypothermia led to a strong increase in [Ca²⁺]ₘ, which was significantly inhibited by dopamine pre-treatment (Fig. 4). Like dopamine, BAPTA-AM prevented mitochondrial Ca²⁺ accumulation. The effect of dopamine on the mitochondrial Ca²⁺ influx could be overcome by ionomycin.
**Respiratory chain inhibitors**

A consequence of mitochondrial Ca$^{2+}$ accumulation is MPT and loss of mitochondrial membrane potential ($\Delta\Psi$). During hypothermia, dopamine prevents the loss of $\Delta\Psi$ \[22\]. We tested if respiratory chain inhibitors (myxothiazol, Na-azide), an inhibitor of ATP synthase (oligomycin) and an uncoupler (CCCP), all of which are known to generate mitochondrial ROS, were able to overcome the protective effect of dopamine or EDTA. Myxothiazol (data not shown) and Na-azide both abrogated the protective effect of dopamine completely (Fig 5). Oligomycin and CCCP were less effective in this regard. It must be stressed that none of these compounds influenced cell viability when tested in similar concentrations on HUVEC that were kept at 37ºC (data not shown). In the presence of EDTA, HUVEC were still protected from hypothermia mediated cell death, even when Na-azide, CCCP or oligomycin were added to the cells (Fig 5).

### Discussion

In the present paper we studied the importance of redox imbalance, changes in calcium homeostasis and depletion of ATP in hypothermia mediated cell death and evaluated the mechanisms by which dopamine protects against these deleterious effects. The main findings of this study are: first, hypothermia leads to a redox imbalance. This is not the result of an increased ROS production but rather due to depletion of SH-reduction equivalents. Second, hypothermia leads to a calcium influx, which in turn results in an increased mitochondrial Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{m}$). Third, hypothermia leads to ATP depletion. Fourth, dopamine pre-treatment of HUVEC renders the cells transiently resistant to hypothermia induced cell death. The loss of SH-reduction equivalents is significantly less, ATP depletion is retarded and mitochondrial calcium accumulation is significantly inhibited under these conditions.

Recently, the role of Ca$^{2+}$ in cold preservation damage of liver endothelial cells and hepatocytes has been questioned \[27\]. It was suggested that cold induced injury occurred predominantly via an iron dependent pathway. Because EDTA, BAPTA-AM and even dopamine, also have the propensity to chelate iron, our results do not unequivocally point out towards a critical role for Ca$^{2+}$ in this process. Yet, if iron chelation is the major mechanism by which dopamine exerts its protective effect, a number of issues have to be addressed. Importantly, it does not explain why dopamine prevents intracellular Ca$^{2+}$ accumulation. Furthermore, it does not explain why Ca$^{2+}$ release from the intracellular stores can overcome the protective effect of dopamine. The protective effect of dopamine is strictly redox dependent \[22\], however, we can not exclude that oxidation of dopamine impairs the ability to chelate iron. Unpublished findings also have shown that protection mediated by dihydroxy phenolic

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**Fig 5.** Influence of respiratory chain inhibitors and ionomycin on dopamine’s protective effect. Prior to cold storage, HUVEC were pre-treated with 25µM of DA for 2hrs or left untreated. Hereafter the cells were subjected to hypothermia for 24hrs in the absence or presence of ionomycin (10µM), oligomycin (10µM), Na-azide (10µM) or CCCP (10µM). In untreated cells hypothermia was performed in the presence or absence of 10mM of EDTA. Supernatants were collected and assessed for LDH release. The results are expressed as mean LDH release ± SD. A total of 4 experiments were performed.
compounds, was only observed when the hydroxyl groups were in ortho- or para- but not in meta-position. While any of these compounds are able to chelate iron, the meta-dihydroxy phenolic compounds cannot be oxidized, and hence cannot donate reduction equivalent. This also emphasizes that iron chelation is unlikely underlying the protective effect of dopamine.

There is convincing evidence that redox potential can influence Ca²⁺ release from the endoplasmatic reticulum (ER) [28]. Koshita [29] found that Ca²⁺ release from the sarcoplasmatic reticulum could be induced by oxidizing compounds and that this was blocked in the presence of GSH. In untreated HUVEC depletion of SH-reduction equivalents occurred already within 4 hrs of hypothermic preservation. Although the mechanism by which this occurred, i.e. oxidation or release from the cell, has not been addressed in this study, it is an eligible condition that could lead to Ca²⁺ release from the ER. Subsequently, Ca²⁺ might enter the cells via store-operated channel, leading to a critical Ca²⁺ concentration in the cytosol [30-32]. A number of observations are in line with this sequel of events. Firstly, at early time-point dopamine completely prevented the depletion of SH-reduction equivalents. Although at 24 hrs also in dopamine treated cells SH-reduction equivalents were partially depleted, this was significant less compared to untreated cells. Secondly the protective effect of dopamine could be abrogated by thapsigargin, a compound that prevents the re-uptake of Ca²⁺ in the ER and hence results in partial depletion of Ca²⁺ from the ER through leakage into the cytosol. Because thapsigargin could not abrogate cell protection when cold preservation was performed in the presence of EDTA, this indicates that the amount of Ca²⁺ released from the ER is not sufficient for cell death and that cell death dependents on additional Ca²⁺ influx.

This study does not argue against an important role for iron in cold preservation injury. In fact, a number of studies already have shown the importance of iron in this respect. Kerkweg [23] have demonstrated that the chelatable iron-pool is increased during hypothermia. Moreover, studies using deferoxamine have clearly demonstrated the beneficial effect of iron chelating on hypothermia mediated cell death [23, 24]. Over-expression of HO-1 has also been shown to be beneficial with respect to hypothermic preservation injury [33]. This is partly mediated via the generation of CO as reviewed by Nakao [34]. CO can bind iron directly and hence might prevent the formation of hydroxyl radicals by Fenton chemistry. Formation of radicals in conjunction with depletion of SH-reduction equivalents will result in a redox imbalance, thereby linking the iron dependent pathway with Ca²⁺ overload.
Since ATP is required to maintain \( \text{Ca}^{2+} \) homeostasis, it can be argued that suppression of mitochondrial activity under hypothermic conditions might be the initial event leading to \( \text{Ca}^{2+} \) overload. Based on time course experiments however, an increased \([\text{Ca}^{2+}]_{\text{m}}\) was evident within 3hrs of hypothermia, while ATP depletion was marginally influenced at this time-point. Although in dopamine treated cells ATP depletion was also observed after 24 hrs of hypothermic preservation, we have previously demonstrated that at this time-point the mitochondrial membrane potential (\( \Delta \Psi \)) was still intact [22]. Inasmuch as respiratory chain inhibitors or uncouplers could overcome the protective effect of dopamine, this was unlikely due to an impaired ATP generation as hypothermia per se is associated with a decreased mitochondrial activity. Nevertheless, these compounds are able to generate mitochondrial ROS production [33], which subsequently might leak to the cytosol, oxidize dopamine and thus abolish its protective effect.

Based on our results and data from the literature we propose the following model of hypothermic preservation injury as depicted in Figure 6. We are aware that this model should not be generalized for all cells or tissues. Nevertheless, it explains why the protective effect of dopamine is redox dependent and why dopamine prevents \( \text{Ca}^{2+} \) overload during hypothermic preservation.

Our model does not include transcription factors, although transcription factor might be activated during pre-treatment with dopamine. We are aware of the studies of Roberts et al [35] showing that cold preservation of HUVEC results in activation of NF-\( \kappa \)B, ERK 1/2 and p38 MAPK pathways. We have previously shown that ERK 1/2 is completely dephosphorylated upon cold storage, suggesting that no activation of this pathway occurs in our model [36]. Similarly, unpublished findings also argue against a role of NF\( \kappa \)B in the protective effect of dopamine. Importantly, proteasome inhibition does not overcome the protective effect of dopamine, although activation of NF-\( \kappa \)B is completely prevent (data not shown). Proteosome inhibitors themselves are also not protecting endothelial cells against cold preservation.

What are the possible implications from this study for organ preservation? One important implication is that during organ preservation cellular calcium entry must be prevented. To avoid the deleterious effects of \( \text{Ca}^{2+} \) accumulation in the cells, \( \text{Ca}^{2+} \) has been omitted from most preservation solutions [37]. However, these solutions are not completely devoid of \( \text{Ca}^{2+} \) since a small amount of this cation is likely to be present in the solvent. Moreover, complete omission of \( \text{Ca}^{2+} \) can lead to opening of unselective cation channels and hence to membrane depolarization. In fact, addition of small amounts of calcium to preservation solutions has proven to be more protective in experimental liver transplantation [38, 39]. Based on the proposed model, prevention of cellular calcium entry can be achieved either by reducing iron mediated oxidative stress or by preventing the occurrence of a redox imbalance. In the present study we have demonstrated that dopamine is capable to prevent redox imbalance. Because this phenomenon is not exclusive for dopamine, but holds true for other hydrophobic dihydroxyphenolic compounds [22], the use of such compounds devoid of hemodynamic action are of potential clinical relevance to prevent hypothermic preservation of organ allografts.

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References


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