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Hypoxia/hypercapnia prevents iron-dependent cold injuries in cord blood stem and progenitor cells

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Abstract

Background. Cold-induced cell injuries are associated with an increase in the cellular labile iron pool (LIP) followed by lipid peroxidation and alteration of mitochondrial function, which lead to cell death. Recently, we showed that incubation in a hypoxic/hypercapnic (HH) gas mixture improved the survival of a population of cord blood hematopoietic progenitors and $CD34^+$ hematopoietic progenitor and stem cells in severe hypothermia. To explain the underlying mechanism, here we test if this HH-induced cytoprotection in cold conditions is associated with the level of LIP and lysosome stability. *Methods.* Cord blood $CD34^+$ cells were incubated in air (20% O_2/0.05% CO_2) or in the hypoxic (5% O_2)/hypercapnic (9% CO_2) atmosphere for 7 days at 4°C and analyzed. *Results.* Incubation in HH condition maintained the day 0 (D-0) level of LIP detected using a bleomycin-dependent method. This was associated with preservation of lysosome integrity and a higher cell survival. Conversely, in the air condition LIP was significantly increased. Also, the presence of a moderate concentration of iron chelator deferoxamine improves the conservation of total $CD34^+$ cells and committed progenitors in air condition. Pre-treatment of $CD34^+$ cells with the lysomotropic agent imidazole induces significant decrease in the lysosomal stability and in all conditions. This is associated with an important decrease of survival of conserved cells and an increase in the cellular LIP level. *Discussion.* Our study showed that HH gas mixture cytoprotection during hypothermia maintains lysosome stability, which enables preservation of the cellular chelatable iron in the physiological ranges. These findings suggest a way to optimize cell conservation without freezing.

Key Words: *$CD34^+$ cells, conservation, hypercapnia, hypothermia, hypoxia, iron, lysosomes*

Introduction

Storage in severe hypothermia (4°C), for more than a few days, triggers cellular injuries. It is generally proposed that the primary cause of damage comes from cold-induced mismatch between adenosine triphosphate (ATP) supply and demand, leading to a cellular energy deficit. Consequently, the failure of ion-motive adenosine triphosphatases (ATPases) is followed by an impairment of ionic balance. This leads to membrane depolarization and an uncontrolled influx of Ca^{2+} through voltage-gated Ca^{2+} channels, resulting in the activation of Ca^{2+}-dependent hydrolases, and in turn provoking further membrane depolarization, uncontrolled cell swelling and, ultimately, cell necrosis [1,2]. These events are potentiated by the behavior of plasma membrane lipids in hypothermia, which undergoes lipid phase transition interfering with membrane function, leading to ion leakage [3].

More recent studies have shown in various cell types that cold-induced cell injuries are associated with an increase in the cellular chelatable labile iron pool (LIP), initiating the formation of reactive oxygen species (ROS) [4-7]. The increase in ROS levels is usually followed by a massive lipid peroxidation, alteration of mitochondrial function and loss of lysosome integrity, which can lead to

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cell necrosis in hypothermia or apoptosis upon rewarming [4].

Hematopoietic stem cells and hematopoietic progenitor cells (HSCs/HPCs) are very sensitive to hypothermia, and their optimal storage conditions (conservation medium, temperature, duration) are critical for successful stem cell transplantation [8].

Surprisingly, the mechanisms of the cold-induced injuries of HSC/HPC have been poorly explored. Recently, we described a method for prolonged cold storage enabling functional preservation of cord blood (CB) HSCss/HPC in a hypoxic/hypercapnic (HH) gas mixture [9,10]. To explain the underlying mechanism, we tested if this HH-induced cytoprotection in cold is associated with the level of LIP and lysosome stability.

Material and methods

$CD34^+$ cell processing

Purification

CB samples delivered (with the mother's approval) to the Cell Therapy Unit of the French Blood Institute, Bordeaux, rejected for banking, were used in experiments. $CD34^+$ cells from umbilical CB were isolated using an immunomagnetic technique (Miltenyi Biotec).

Conservation

$CD34^+$ cells (5×10^4/mL) were plated in Stemα S3 (Stem Alpha SA; Saint Genis), incubated in air (20% O_2/0.05% CO_2) or in the hypoxic (5% O_2)/hypercapnic atmosphere (9% CO_2) for 7 days at 4°C. All of the tests were performed with purified (day [D]-0) and stored $CD34^+$ cells after 7 days at 4°C (D-7). To obtain the desired gas mixtures, the open flasks with cell suspension were incubated in an O_2 and CO_2 culture chamber equipped with O_2 and CO_2 controllers (PRO-OX and PRO-CO_2; Biospherix) for 1 h at room temperature then plugged and transferred to 4C° conditions for 7 days. In some variants of the experiment, deferoxamine (DFX; Sigma Aldrich) was added to the conservation medium. For the estimation of the effect of the lysosomotropic agent on the cell conservation, cells were pre-incubated for 1 h at 37°C with 250 mmol/L imidazole (Sigma Aldrich) in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific) supplemented with 2% fetal calf serum (HyClone Perbio) and 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Sigma Aldrich), washed and resuspended in conservation medium before transfer to 4°C. Cells were harvested at D-7 and then analyzed.

Apoptosis assay

Apoptosis was determined with Annexin V-Fluorescein isothiocyanate (FITC) kit (Beckman Coulter) following the manufacturer's protocol. Briefly, 10^5 $CD34^+$ cells were labelled with Annexin V-FITC solution (AnnV) and propidium iodide (PI; 10 mg/mL) for 15 min at 4°C, then washed in Phosphate-buffered saline and analyzed with a flow cytometer (FACSCanto II; Beckton Dickinson).

Hematopoietic committed progenitor determination: colony-forming cell assay

The committed progenitor colony-forming cells (CFCs) (colony-forming unit–granulocyte, monocyte [CFU-GM] plus burst-forming unit–erythroid [BFU-E]) were assayed as previously described [9]. Briefly, D-0 and D-7 $CD34^+$ cells (250 cells/mL and 600 cells/mL, respectively) were seeded in methylcellulose Stem Alpha-1D (Stem Alpha; Stem Alpha Saint Genis) supplemented with recombinant human stem cell factor (SCF), recombinant human interleukin 3 (IL-3), recombinant human erythropoietin (EPO) and recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF). After 14 days of incubation, CFCs were counted.

Ferritin content measurement

The assay was performed using 10^5 $CD34^+$ cells harvested at D-0 or D-7 conservation in air or HH conditions. Total proteins were extracted with Radioimmunoprecipitation assay buffer (RIPA) buffer (50 mmol/L Tris/HCl pH 7.5, 150 mmol/L NaCl, 1% Triton X 100, 0.5 Na-deoxycholate, 0.1 mmol/L cocktail of protease inhibitors, 1 mmol/L ethylenediaminetetraacetic acid [EDTA]); the reagents were purchased from Sigma Aldrich and quantified with a bicinchronic acid (BCA; Thermo Fisher Scientific)–based method. Ferritin content was determined in protein extracts according to the manufacturer's instructions (Human Ferritin enzyme-linked immunosorbent assay enzyme-linked immunosorbent assay [ELISA] Kit; Sigma Aldrich). Quantity of ferritin was determined with biotinylated detection antibodies in the presence of horseradish peroxidase–conjugated streptavidin. Trimethylbenzidine substrate was added and the intensity of absorbance of the colored product was measured at 450 nm (Evolis; Biorad).

Lysosome integrity analysis

Lysosome stability was determined using acridine orange (AO; Molecular Probes) staining, as showed

previously [11]. Briefly, 10^5 $CD34^+$ cells were treated with AO (25 nmol/L) at 37°C for 30 min. To exclude dead cells, 7-amino-actinomycin (7-AAD) was added (BD Bioscience). After rinsing step, pellet was resuspended in phosphate-buffered saline (Lonza) and analyzed with a flow cytometer. Excitation was obtained with argon laser (488 nm) and the acquisition was effected in the phycoerythrin emission spectra using filter 585/40 nm.

Bleomycin-based labile iron determination

LIP was measured using bleomycin-detectable iron assay [12]. All of the solutions used in the assay were Chelex 100 (Sigma Aldrich) treated to eliminate residual iron. All of the reagents were purchased from Sigma Aldrich, unless being stated in the article otherwise. $CD34^+$ cells, harvested at D-0 and D-7 of conservation, were washed with Hank's Balanced Salt Solution (Thermo Fisher Scientific) and then lysed (TRIS/HCl at 20 mmol/L; pH 7.4) with TRITON X-100 1%. Cell lysates were mixed with 0.25 mL of calf thymus DNA (1 mg/mL), 0.05 mL of 5 mmol/L $MgCl_2$, 0.025 mL of bleomycin sulphate (1 mg/mL; Calbiochem) and ascorbic acid (8 mmol/L). After incubation at 37°C for 30 min in a shaking water bath, malondialdehyde was produced proportionally to the quantity of Fe^{2+} ions. The reaction was stopped with 0.5 mL of 0.1 mol/L EDTA. The contents were then mixed with 0.5 mL of 1% thiobarbituric acid (in 50 mmol/L NaOH) and 0.5 mL 25% HCl, vol/vol and incubated at 95°C to transform the malondialdehyde into a colored product. Intensity of absorbance was read at 532 nm (Specord; Analytik Jena). Values were calculated using 0.10 mL of a 0.25−20 μmol/L $FeCl_3$ as a standard.

Statistical analysis

The Wilcoxon-Mann-Whitney comparison test was used to examine the statistical significance of the differences between the various experimental conditions ($P < 0.05$ was considered statistically significant).

Results

Conservation in HH maintains LIP at the steady-state level

The LIP is thought to be involved in causing cell death during hypothermia [5]. To examine if HH induces the same phenomenon, we measured bleomycin-dependent LIP in our conditions [13]. Our results showed that a 7-day exposure to HH maintained the D-0 level of LIP detected using a bleomycin-dependent method. This D-0 content of LIP cells we refer to as the physiological (steady-state) LIP level. Conversely, in the atmospheric air usually used for cell storage at 4°C, cellular LIP was significantly increased compared with D-0 level (Figure 1A).

The presence of an iron chelator in the conservation medium DFX improved the maintenance of $CD34^+$ cells in a dose-dependent manner in air condition, indicating that iron-dependant injuries underline cell degradation in the cold. However, when in the HH condition, where LIP is maintained in the physiological range, the presence of DFX did not significantly increase total cell maintenance (Figure 1B).

In addition, committed progenitors (CFCs) are significantly better preserved in the presence of a moderate concentration of DFX in air condition. But, stronger iron chelation by a high concentration of DFX abrogates the maintenance of CFCs, especially erythroid progenitors BFU-E, and this in all experimental conditions. This implies that iron is indispensable for CFC survival. In addition, our results suggest that the increase of LIP above this physiological range, as well as its decrease to less than steady-state level induced by DFX-chelation, abrogates the survival of hematopoietic progenitors (Figure 1C).

Conservation in HH maintains lysosome integrity and ferritin content

Hypothermic cell injuries might be attenuated by the chelation of LIP with a high-iron storage protein ferritin-dependant mechanism [13]. To determine if the HH condition displays its cytoprotective effect via the ferritin-dependant mechanism, we examined cellular ferritin content. Our results showed that at D-7 there is no statistical difference between experimental conditions, with results similar to the ferritin level detected in the D-0 $CD34^+$ cells (Figure 2A).

Lysosomes contain redox active iron that could leak into the cytoplasm when the lysosomal integrity is altered. The release of this iron and hydrolytic enzymes may contribute to subsequent cell death and cold-induced cell injuries [14]. In view of this, lysosome integrity upon incubation in hypothermia was analyzed using AO, fluorescent dye taken up by proton trapping and retained inside acidic organelles such as lysosomes [11]. After 7 days of incubation in hypothermia and air condition, mean fluorescence intensity (MFI) of AO was significantly diminished compared with the D-0 level, indicating important lysosome degradation. By contrast, in the HH condition, the MFI was similar to D-0 level of $CD34^+$ cells, indicating that lysosomes are protected from degradation when hypothermic incubation takes place in the HH mixture (Figure 2B).

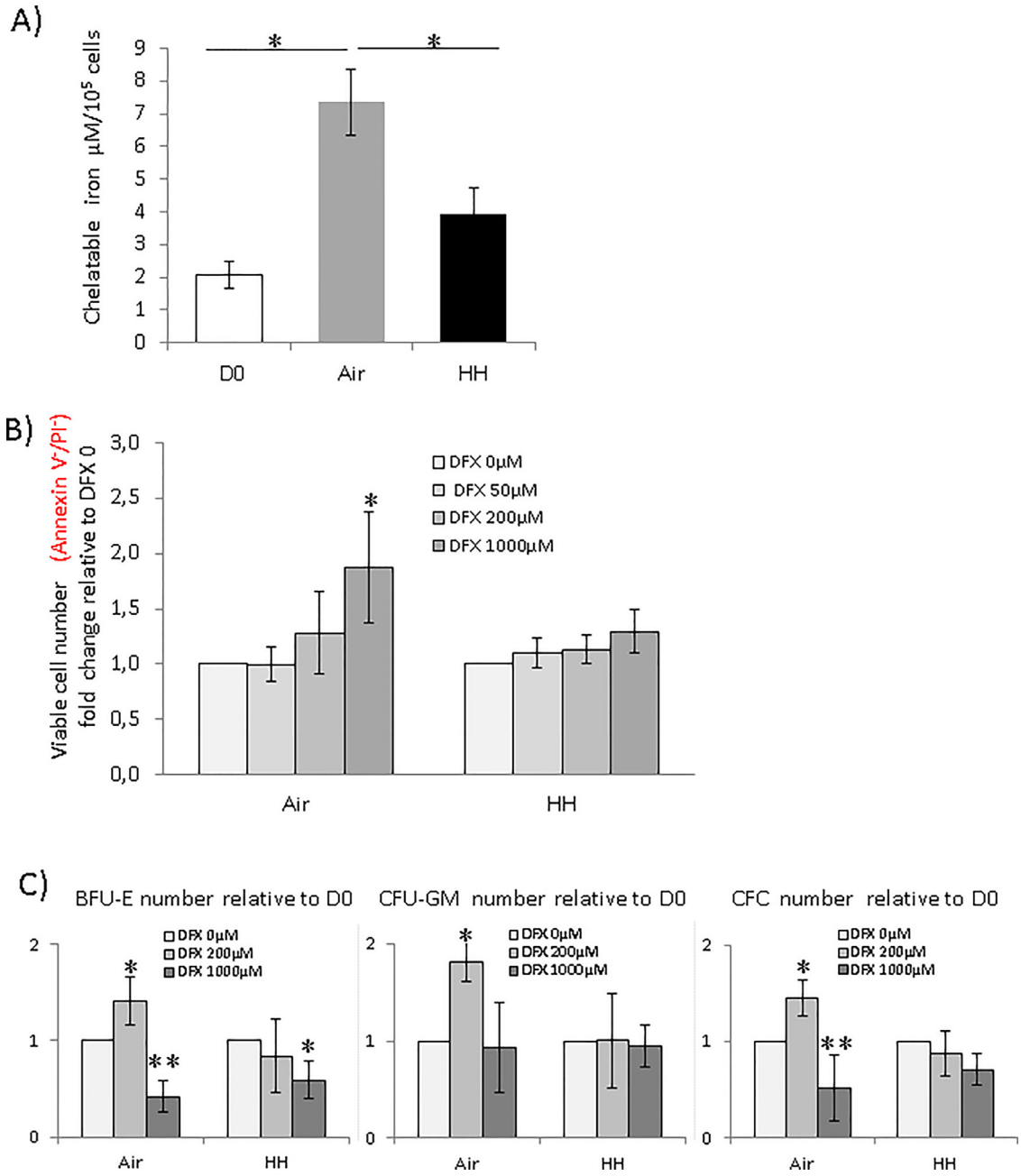
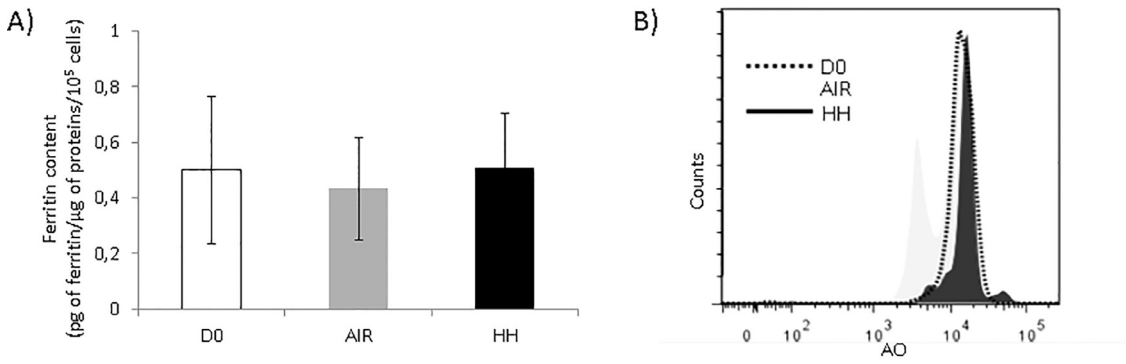


Figure 1. Chelatable iron-dependant conservation in hematopoetic progenitors in hypothermia. (A) Bleomycin-detectable LIP is estimated in $CD34^+$ cells at D-0 and D-7 of incubation at 4°C in air or HH gas mixture. The bars are presented as a mean ± SD of eight independent experiments. (B) Conservation of $CD34^+$ cells presented as the average fold change in the number of viable cells ($Ann\text{-}V^-/PI^-$) detected in the presence of DFX relative to the number of cells detected in the same experiment without DFX. (C) Conservation of the erythroid, granulocyte-monocyte and total committed progenitors in the presence of the growing concentrations of DFX in air or HH gas mixture. The bars are presented as a mean number of the progenitors relative to D-0 level ± SD of six independent experiments. The asterisks indicate a significant difference at, $p < 0.05$ (*), $p < 0.01$ (**); Wilcoxon-Mann-Whitney.

Preservation of lysosome integrity using HH mixture during hypothermia correlates with cell survival and stabilization of LIP level

To discern the relationship between the HH-mediated maintenance of lysosome integrity and cell survival during hypothermic storage, we pre-treated $CD34^+$ cells with a lysomotropic agent, imidazole. Our results showed that imidazole induces a significant decrease in lysosomal stability after 7 days of incubation, and this in a similar manner in air and HH conditions compared with D-0 (Figure 3A). This alteration of lysosome integrity correlates with



Condition	D0	AIR	HH
MFI AO	13310 ± 1800	10015** ± 1480	13830 ± 1324

Figure 2. Ferritin content and lysosome integrity in CD34$^+$ cells during hypothermia. (A) Level of ferritin is determined in CD34$^+$ cells at D-0 and D-7 of incubation at 4°C in air or HH gas mixture. (B) Stability of lysosomes is evaluated using AO using flow cytometry in CD34$^+$ cells at D-0 and D-7 of incubation at 4°C in air or HH gas mixture presented in the histogram. One representative of five independent experiments is shown. AO mean fluorescence intensity (MFI) is presented as the mean ± SD. The asterisks indicate a significant difference at $p < 0.01$ (**) vs D-0 condition; Wilcoxon-Mann-Whitney test.

the significant increase in LIP level compared with D-0 in both conditions (Figure 3B). Also, it coincides with a significant decrease in survival of conserved CD34$^+$ cells in either gas mixture (Figure 3C).

Discussion

Our study indicates that cytoprotection in HH against severe hypothermia is mediated by the stabilization of lysosome integrity and that iron-dependant cold-induced injuries are critical for the survival of CB CD34$^+$cells. We show here that this issue is valid for the total CD34$^+$ cell population (consisting mainly of various hematopoetic progenitors and rare stem cells [15]) as well as its CFC fraction.

Lysosomes contain redox-active iron and a wide spectrum of hydrolytic enzymes, which could provoke death when released into the cytoplasm [16]. Also, lysosomes have been identified as being particularly susceptible to hypothermia; a decrease in lysosome integrity is consistent with cold-induced loss in cell viability [14]. These observations have been proved for the various cells types and organs [14,17–19] and here we reported similar data for the hematopoietic progenitors.

In our study, when lysosomal stability was compromised, the cytoprotective effect of HH during hypothermia was significantly abrogated, which correlates with an increase in LIP and a decrease in cell viability (Figure 3).

Our results fit with previously published data showing that one of the consequences of alteration of lysosome integrity on cell survival during hypothermia is the release of chelatable iron [5,14,20]. In presence of ROS, which were shown to have increased in hypothermic conditions (other authors' and our data [5,9]), the oxidative iron-dependant Fenton-type reactions can take place [5]. It was reported that this might result in massive lipid peroxidation that, in turn, attacks the lysosomes but also other cell membrane structures and leads to cold-induced injuries [16].

Interestingly, our work suggests that LIP, but not oxidative stress, is particularly important in the occurrence of cold-induced injuries and cell death in hematopoietic progenitors. More specifically, we showed recently that HH cytoprotection is ruled out despite sustained ROS production [9]. Also, in this study, the maintenance of LIP within the physiological range is crucial for the survival of hematopoetic progenitors because its increment above steady-state level abrogates HH-mediated cell protection in hypothermia (Figure 3).

Thus, we propose that functional preservation of CD34$^+$ cells in hypothermia by HH involves stabilization of the lysosome membrane, which prevents release of LIP that could provoke deadly cold-induced injuries in the presence of hypothermia-produced ROS. This raises the question of how the HH gas mixture stabilizes the lysosome membrane in the first place. A possible explanation lies in the fact that hypercapnic condition creates a slightly acidic environment (intracellular and extracellular) when in hypothermia; we have shown this previously [9]. In hypercapnic condition, CO_2 diffuses across the membrane, enabling a permanent load of carbonic acid and protons in the cytosol. In these

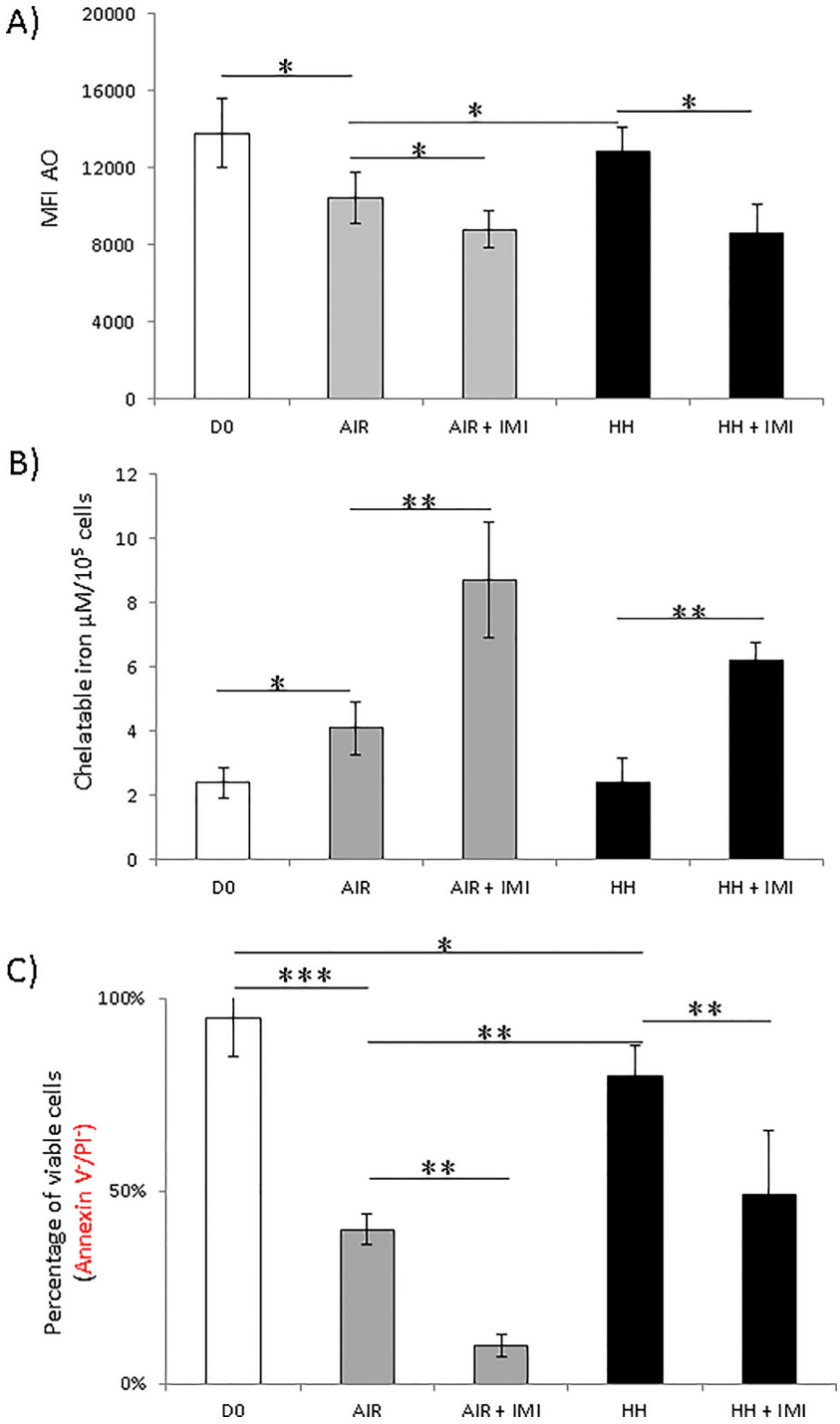


Figure 3. Conservation of $CD34^+$ in hypothermia pre-treated with imidazole. (A) AO staining of lysosomes in $CD34^+$ cells at D-0 and D-7 of incubation at 4°C in air or HH gas mixture. (B) The bleomycin-detectable LIP is estimated in $CD34^+$ cells at D-0 and D-7 of incubation at 4°C in air or HH gas mixture. (C) $CD34^+$ cell survival after 7 days of incubation at 4°C in air or HH gas mixture. Cell survival is presented as the percentage of viable cells ($Ann\text{-}V^-/PI^-$). The bars are presented as a mean ± SD of five independent experiments. The asterisks indicate a significant difference at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***); Wilcoxon-Mann-Whitney test. IMI, imidazole.

conditions, the plasma membrane lipid bilayer is protected from cold-induced injuries [7,9]. In addition to the plasma membrane, this protecting effect could be applied to all other cellular membrane structures including the lysosome.

Thus, according to our previous work and these findings, we suggest that preservation of $CD34^+$ cells during hypothermic storage is mediated by the double action of HH gas mixture; hypercapnia diminishes the occurrence of cold-induced injuries, while

hypoxia prevents apoptosis upon rewarming after the transfer to 37°C [9,10,21].

Because LIP has a critical role in cell survival during hypothermic incubation of cultured cells, improved cell preservation was accomplished by the utilization of conservation medium supplemented with iron chelators [5,7]. In this study, DFX added in the conservation medium improved cell viability only in the conditions where LIP was above steady-state (D-0) level in $CD34^+$ cells (Figure 1).

At 37°C, DFX is internalized by endocytosis and localized exclusively in lysosomes, where it acts as a chelator of intralysosomal iron, stabilizing the lysosomes [22]. At 4°C, endocytosis is blocked and the DFX remains in the extracellular compartment, where it removes the iron from the extracellular space. But, because labile iron could pass through the membrane, the presence of DFX in the extracellular compartment [23] could diminish intracellular iron too.

With our method, the DFX is added to the conservation medium during the preparation phase dedicated to obtaining the desired gas mixture, which is done at room temperature. At this temperature, DFX could slowly enter the intracellular compartment by endocytosis but could not be trafficked to the lysosomes [24]. Thus, with this method, the DFX could capture the iron from the intracellular as well as the extracellular environment.

On the other hand, our results showed that a decrease below steady-state level of LIP with the highest dose of DFX is not compatible with the survival of commited hematopoetic progenitors in either gas mixtures (Figure 1C). These results were expected since it has been shown in the model of anemic Belgrade laboratory (b/b) rat [25] that severe deficiency of intracellular iron provoked by the mutation of the iron transport protein Nramp2 [26] blocks the proliferation of the hematopoietic progenitors (in this case colony-forming unit–spleen [CFU-S]) [27]. This was completely bypassed by the treatment of b/b rats with a high dose of molecular iron or a non-toxic dose of hemin [25].

With our method, incubation in HH preserves functional CB stem and progenitor cells significantly more efficiently than other models of cold storage without freezing [9,10]. Here we revealed that lysosome stability and iron-dependant cold-induced injuries are the critical issues for hematopoietic progenitor cold preservation. In this regard, we think that the addition of iron chelators or stabilizers of the lysosome membrane are the way to optimize CB storage survival. Our approach could be an alternative to freezing in clinical practice in some situations. This should enable avoiding cryopreservation of the hematopoietic grafts intended for transplantation shortly after collection (some cases of autologous and intra-familiar cord blood transplantation) or for long-distance transport. Also, it would facilitate organization and improve results of autologous or allogenic transplantation procedures.

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Disclosure of interests: The authors declare that they have no conflicts of interest.

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