Low-oxygen and high-carbon-dioxide atmosphere improves the conservation of hematopoietic progenitors in hypothermia

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BACKGROUND: During short-term storage of hematopoietic cells (HCs) at 4°C a substantial decline in number and in functional capacity of progenitors occurs after 3 days. We hypothesized that physiologic O_2 and CO_2 concentrations of hematopoietic tissue microenvironment (approx. 3% O_2 and approx. 6% CO_2) could improve cell viability and functionality during storage at 4°C.

STUDY DESIGN AND METHODS: Mobilized peripheral blood (PB) CD34+ cells from multiple myeloma or non-Hodgkin's lymphoma patients were stored in flasks containing air (approx. 20% O_2 and approx. 0.05% CO_2) or 3% O_2/6% CO_2 atmosphere, for 3, 5, and 7 days at 4°C. The total number of cells, the number of cells in G0 or G1 phase of cell cycle, and the apoptosis rate were determined. The functional capacity of stored cells was assessed by the capacity of progenitors to form colonies in methylcellulose (colony-forming cells [CFCs]) and of stem cells to repopulate the bone marrow (BM) of immunodeficient mice (SCID-repopulating cell [SRC] assay).

RESULTS: The total number of viable cells and cells in G1 phase as well as the number of total CFCs were significantly higher at 3% O_2/6% CO_2 than in air at all time points. Cells in G0 phase and SRC were equally preserved in both conditions.

CONCLUSION: Atmosphere with low O_2 and high CO_2 concentration (3% O_2/6% CO_2) in hypothermia (+4°C) during 7 days of storage prevents cell damage and preserves a high number of functional HSCs and progenitors mobilized in PB by granulocyte–colony-stimulating factor.

Cryopreservation is actually the only method for mid- and long-term storage of hematopoietic cells (HCs). However, the possibility of "clumping" during thawing as well as several side effects after transplantation of thawed HCs including life-threatening anaphylactic reaction due to the presence of cryoprotectors, represents rare but serious consequences.[1,2] An optimal system that would enable the short-term (7 to 10 days) liquid storage of hematopoietic stem cells (HSCs) and progenitor cells (HPCs) at 4°C could, in some cases, facilitate organization and improve results of autologous or allogenic transplantation procedures.

Short-term liquid storage of HCs at 4°C induces less perturbation in cell microenvironment than cryopreservation. However, their storage over 3 days induces substantial loss in number and functional capacity of progenitors.[3-5] The data on improvement of fresh blood and separated blood cell storage at 4°C are complex and controversial. They include evaluation of different temperature and storage solution composition,[3,5,6] the effect of air in storage vials, and gas permeability of collection bags.[7-9]

Storage at 4°C, for example, in hypothermia results in a specific metabolic state termed "hypometabolism."

ABBREVIATIONS: AnV = annexin V; BM = bone marrow; FC = flow cytometry; HC(s) = hematopoietic cell(s); MC = methylcellulose; PB = peripheral blood; ROS = reactive oxygen species; SRC(s) = SCID-repopulating cell(s).

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Reversible hypometabolic state of many invertebrates, lower vertebrates, and hibernating mammals (during the decrease of ambient temperature) is associated with a decrease in O_2 availability and an accumulation of CO_2.[10] Nonhibernating rodents enter in hypometabolic state and can survive otherwise lethal short-term hypothermia when intubated with a gas mixture containing O_2 and an increased concentration of CO_2[11,12] that could be explained by a decrease in O_2 consumption and sparing of energy substrate during cooling by hypercapnia.[13] Moreover, in ex vivo systems, short- and long-term preservation of different mammalian cells is improved by addition of antioxidant molecules.[14,15] Carbon dioxide by itself has the property to suppress production of reactive oxygen species (ROS).[16] Altogether, these data lead to the hypothesis that low O_2 and moderately high CO_2 atmosphere could improve in vitro HC survival (especially of stem and progenitor cells) in hypothermia.

This study focuses on the effect of low O_2 and moderately high CO_2 atmosphere (tissue microenvironment) in storage flasks, on mobilized peripheral blood (PB) CD34+ cell survival and apoptosis, clonogenic efficiency of progenitors, and maintenance of stem cell activity during 1-week-long hypothermia. Our results, based on the analysis of 35 studied patients' samples, demonstrated a positive impact of a 3% O_2/6% CO_2 atmosphere with respect to the air (20% O_2/0.05% CO_2) on preservation of CD34+ cells and colony-forming cells (CFCs), as well as that the SCID-repopulating cells (SRCs) were well maintained in both storage conditions.

MATERIALS AND METHODS

Cells

After standard mobilization protocol (filgrastim, 5 μg/kg/day) in multiple myeloma and non-Hodgkin's lymphoma patients, PB CD34+ cells were collected in a nucleated cell fraction using standard leukapheresis procedure.[17] The samples originating from patients (n = 35) with a range of PB CD34+ cells between 2 and 800/μL PB entered the study. The CD34+ cells were selected from the mononuclear cell (MNC) fraction obtained by a ficoll separation from leukapheresis products using indirect CD34+ selection kit (Miltenyi Biotec, Paris, France). Briefly, the first MNC incubation (15 min at 4°C) with blocking reagent and anti-CD34 was followed by washing and second incubation with microbeads (15 min at 4°C) according to the manufacturer's instructions. Each time, cell separation on magnetic columns was repeated twice. Cell purity assessed by flow cytometry (FC; FACSCalibur, Becton Dickinson, San Jose, CA) using an anti-CD34 (clone HPCA2-PE; Becton Dickinson) always exceeded 88%.

CD34+ storage at 4°C and pH measurement

A defined number of CD34+ cells (50×10^3 per mL) were resuspended in serum-free, cytokine-free liquid storage medium (Stem alpha.S3, Stem Alpha SA, Saint Genis l'Argentière, France) and distributed in 50-mL flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) coated overnight with 2 mL of 4% human albumin (Vialebex, LFB, Courtaboeuf, France). The flasks were kept at room temperature for 45 minutes and exposed to air (laminar) or to 3% O_2/6% CO_2 obtained in a chamber provided with gas controllers (PRO-OX and PRO-CO2, Biospherix, Redfield, NY). This gas-exposition length has been chosen on the basis of the preliminary experiments demonstrating an equilibration of gas mixture in liquid (dissolved) and in gas (not dissolved) phases (on the basis of a pH measurement and a change of color of the medium indicator). Once closed rapidly, flasks were stored at 4°C for 3, 5, and 7 days. The endpoint of analysis (Day 7) was chosen on the basis of preliminary experiments demonstrating nearly total disappearance of progenitors (CFCs) in flasks with air. After defined time points (Days 0, 3, 5, 7), cell counts (trypan blue exclusion test), progenitor assay, and in vivo repopulating capacity assays were performed.

Progenitor assay

The committed progenitor CFCs (colony-forming unit–granulocyte-macrophage [CFU-GM] plus burst-forming unit–erythroid [BFU-E]) were detected with a ready to use methylcellulose (MC) kit (Stem alpha.1D, Stem Alpha SA) immediately after CD34+ selection (Day 0) and after 3, 5, and 7 days of conservation. The cells were seeded in MC cultures at 10^3 cells per mL.

All cultures were incubated for 14 days in 5% CO_2, at 37°C, and at 100% humidity (Incubator Stericult 200, Forma Scientific, Inc., Majetla, OH) and the colonies (>50 cells) were enumerated on an inverted microscope (Leica, Wetzlar, Germany).

Detection of stem cells on the basis of their in vivo repopulating capacity (SRC assay)

Before transplantation, NOG female mice (central animal keeping facility of the University Victor Ségalen, Bordeaux, France) received preparative conditioning consisting of intraperitoneal injection of an injectable form of busulfan (Busilvex, Pierre Fabre, Boulogne, France) according to the previously established protocol.[18] Briefly, in a 24-hour period, mice received two busulfan doses of 25 mg/kg. After 7 days of conservation, the cell content of each flask was washed twice in phosphate-buffered saline (PBS) with 1% human albumin (Vialebex, France) and resuspended in 200 μL of bIM (Biochrom AG, Berlin, Germany). Cells

were transplanted (intravenously) 24 hours after the last busulfan injection. After 6 to 7 weeks, the animals were killed, and femoral bone marrow (BM) cells were flushed and analyzed by FC (FACSCalibur, Becton Dickinson) for the presence of human CD45 (fluorescein isothiocyanate [FITC]), CD33 (allophycocyanin), and CD19 (phycoerythrin; monoclonal mouse anti-human antibodies, Becton Dickinson, Pont-de-Claix, France). BM cells of a noninjected mouse incubated with anti-human CD45 were used as a control. To detect the human progenitors among mice BM cells, 12×10^3 human CD45+ BM cells were seeded per mL of MC (Stem alpha.1, Stem Alpha SA) supplemented with 10% human plasma, 25 ng/mL recombinant human (rHu) interleukin-3 (PeproTech, Rocky Hill, NJ), 25 ng/mL rHu granulocyte-monocyte–colony-stimulating factor (R&D Systems Europe, Lille, France), 50 ng/mL rHu stem cell factor (Amgen, Neuilly-sur-Seine, France) and 3 U/mL rHu erythropoietin (Tebu-Bio, Le Perray, France).

Apoptosis assay

Apoptosis was assessed after 3, 5, and 7 days of conservation, with annexin V (AnV)-FITC kit (Beckman Coulter, Roissy, France) following the manufacturer's protocol. Briefly, cell suspension (5×10^4/100 µL) was labeled with 1 µL of annexin and 5 µL propidium iodide (PI) and incubated for 15 minutes at 4°C. After the buffer solution was added, FC was performed (FACSCalibur, Becton Dickinson, San Jose, CA). This technique allows to detect the expression of AnV on viable cells (PI^-; considered as "early apoptotic events") and on death cells (PI^+) still expressing the AnV (considered as "late apoptotic events"), as well as to distinguish the apoptosis-free viable cells (AnV^-/PI^+), whose number, compared to Day 0, values is the direct measure of cell survival, that is, maintenance.

Cell cycle assay

Cell cycle of selected CD34+ cells on Day 0 and of cells after 3, 5, and 7 days of conservation was analyzed using PI and anti–Ki-67 (DAKO A/S, Glostrup, Denmark). The nuclear fixation of PI was used to estimate the DNA content and the expression of the protein Ki-67 as a marker of active cell cycle (G1, SG2M). After conservation, cells were washed (PBS, Ca^{2+}/Mg^{2+} free, 10 mmol/L ethylenediaminetetraacetate, 5% fetal calf serum, and 0.05% azide), fixed, and permeabilized for 30 minutes at 4°C in a solution containing 2% formaldehyde, 0.02% saponin, and 10 mmol/L HEPES. Cells were washed twice and stained with an anti Ki-67–FITC antibody or with the corresponding isotypic control for 30 minutes. After being washed, cells were labeled with PI and analyzed by FC (FACSCalibur, Becton Dickinson).

Statistical analysis

Results were presented as the mean ± standard error (SE). Absolute values or normalized values were given (preservation index). Significance of differences between two groups was determined by the two-tailed Student's t test. Differences with p value of less than 0.05 were considered significant and shown in figures. All statistical analyses were carried out in a computer program (Microsoft Excel, Microsoft Corp., Redmond, WA).

RESULTS

The atmosphere of 3% O_2/6% CO_2 favors preservation of CD34+ cells and CFC stored at 4°C

At 3% O_2/6% CO_2, the number of viable CD34+ cells (Fig. 1A) decreased during the 3-day storage and was maintained at this level (preservation index, 0.6 to 0.8) during the next 4 days of storage. In the same condition, the number of CFCs (Fig. 1B) increased on Day 3 due to an increase of CFU-GM (Fig. 1C) but not BFU-E (Fig. 1D). After 5 days of conservation the number of CFU-GM was in the range of Day 0 values (Fig. 1C) while the number of BFU-E decreased (0.75 of Day 0 values; Fig. 1D). This decline continued since on Day 7 approximately 70% of CFU-GM and 50% of BFU-E were still present compared to Day 0. The storage of cells exposed to air resulted in a more pronounced and progressive decline of all parameters investigated (Figs. 1A-1D).

Better preservation of CD34+ cells, as well as CFCs at 3% O_2/6% CO_2, was consistent with finding that CD34+ cell apoptosis in this condition was less prominent than when cells were exposed to air (Figs. 2A-2C). After equilibration of gas and liquid phase in storage flasks and on Days 3, 5, and 7, pH was found to be much lower in suspension exposed to 3% O_2 /6% CO_2 than in that exposed to air (Table 1).

Both air and 3%O_2/6% CO_2 atmosphere maintain the stem cell activity of CD34+ cells stored at 4°C

The number of human CFCs and the percentage of cells expressing human CD45, CD33, and CD19 antigens in BM of mice transplanted 6 to 7 weeks before with cells stored for 7 days showed that the storage in both gas conditions preserved SRC capacity (Figs. 3A-3D). The degree of human chimerism in the murine BM when animals were grafted with the cells conserved at 3% O_2/6% CO_2 atmosphere (Figs. 3A-3D) was comparable to that of Day 0 (fresh) CD34+ cells (not shown) and tends to be higher compared to results obtained at air (9010 ± 2047 vs. 3956 ± 3711 for CFCs and 16.9 ± 0.6% vs. 10.0 ± 3.0%; injected Day 7 equivalent of 150×10^3 Day 0 CD34+ cells; Figs. 3A-3D).

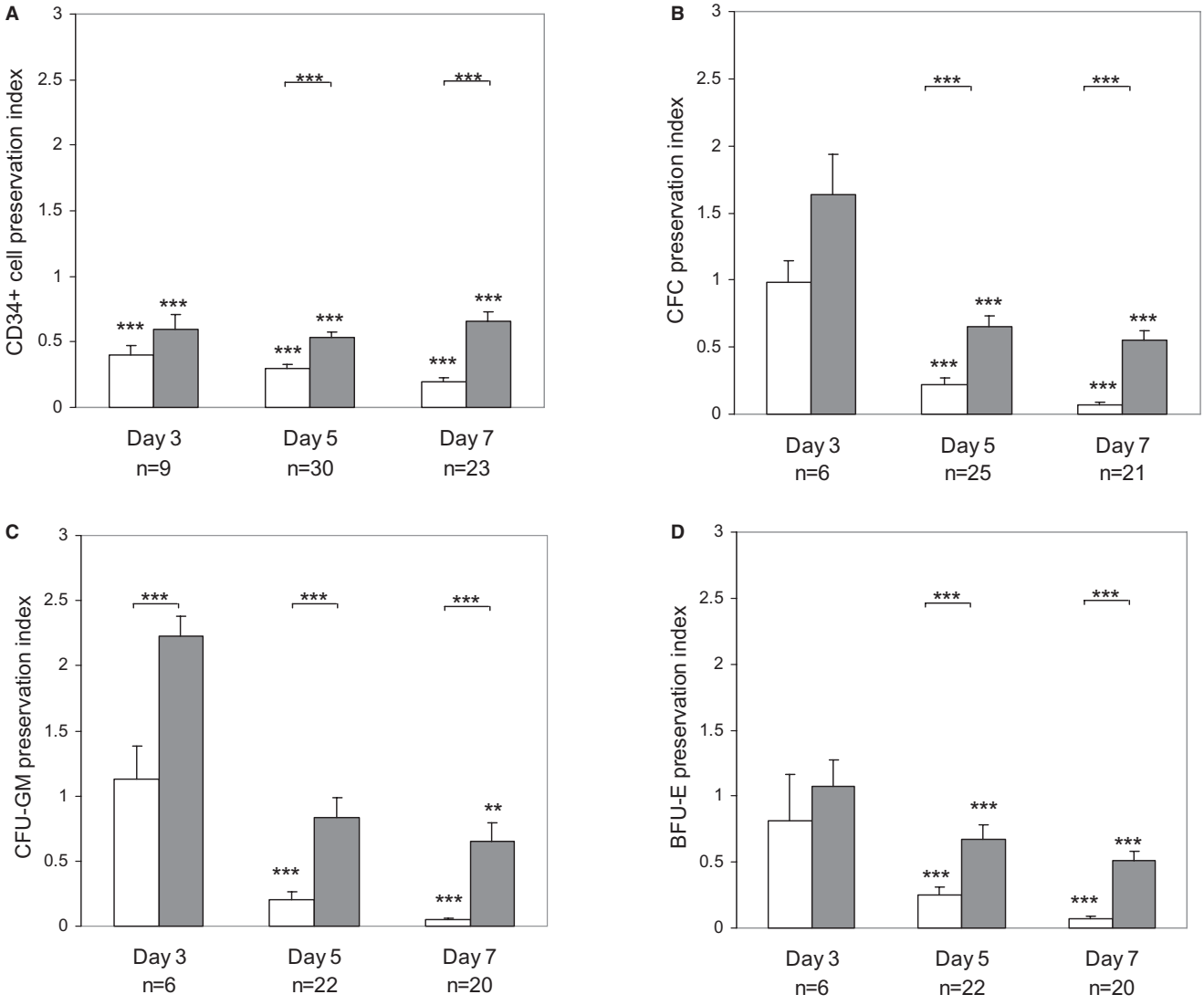

Fig. 1. Preservation index of CD34+ cells (A), total CFCs (B), CFU-GM (C), and BFU-E (D), in the course of conservation in hypothermia in air (□) and at 3% O_2/6% CO_2 (■). Preservation Index 1 represents the number of CD34+ cells or CFCs on Day 0. Data are reported as mean ± SE and the differences between Day 0 and storage at each time point and condition analyzed are given in the form of asterisks on the top of the bars (*p < 0.05; **p < 0.01; ***p < 0.001).

The atmosphere of 3% O_2/6% CO_2 at 4°C preferably maintains the cells in G1 phase of cell cycle

The majority of CD34+ cells mobilized in PB (82.6 ± 1.8 %) were in G1 phase and 16.7 ± 1.6% were in G0 phase of cell cycle. Neither of gas conditions significantly affected cells in G0 phase (Ki-67/IP2n; Fig. 4A). A significant decrease (of approx. 35%) in number of CD34+ cells in G1 phase of cell cycle occurred after 3 days of storage at 3% O_2/6% CO_2, but thereafter their number remained stable until Day 7 (Fig. 4B). In contrast, the number of CD34+ cells in G1 phase of cell cycle stored at air decreased persistently to become on Day 7 five times lower than at 3% O_2/6% CO_2 and 10 times lower than on Day 0 (Fig. 4B). The starting number of cells in SG2M phase was too low to be conclusive (<1%) and was not taken into consideration.

DISCUSSION

Our results demonstrated that O_2/CO_2 concentrations close to those in hematopoietic tissues (3% O_2/6% CO_2) were beneficial for CFC preservation and CD34+ cells viability stored at 4°C in defined storage media. The preservation of more than 60% HPCs and approximately 70% Day 0 CD34+ cells over a 7-day period provided some of the best results obtained among experiments with purified CD34+ cell populations,[6,19] whole apheresis product,[4]

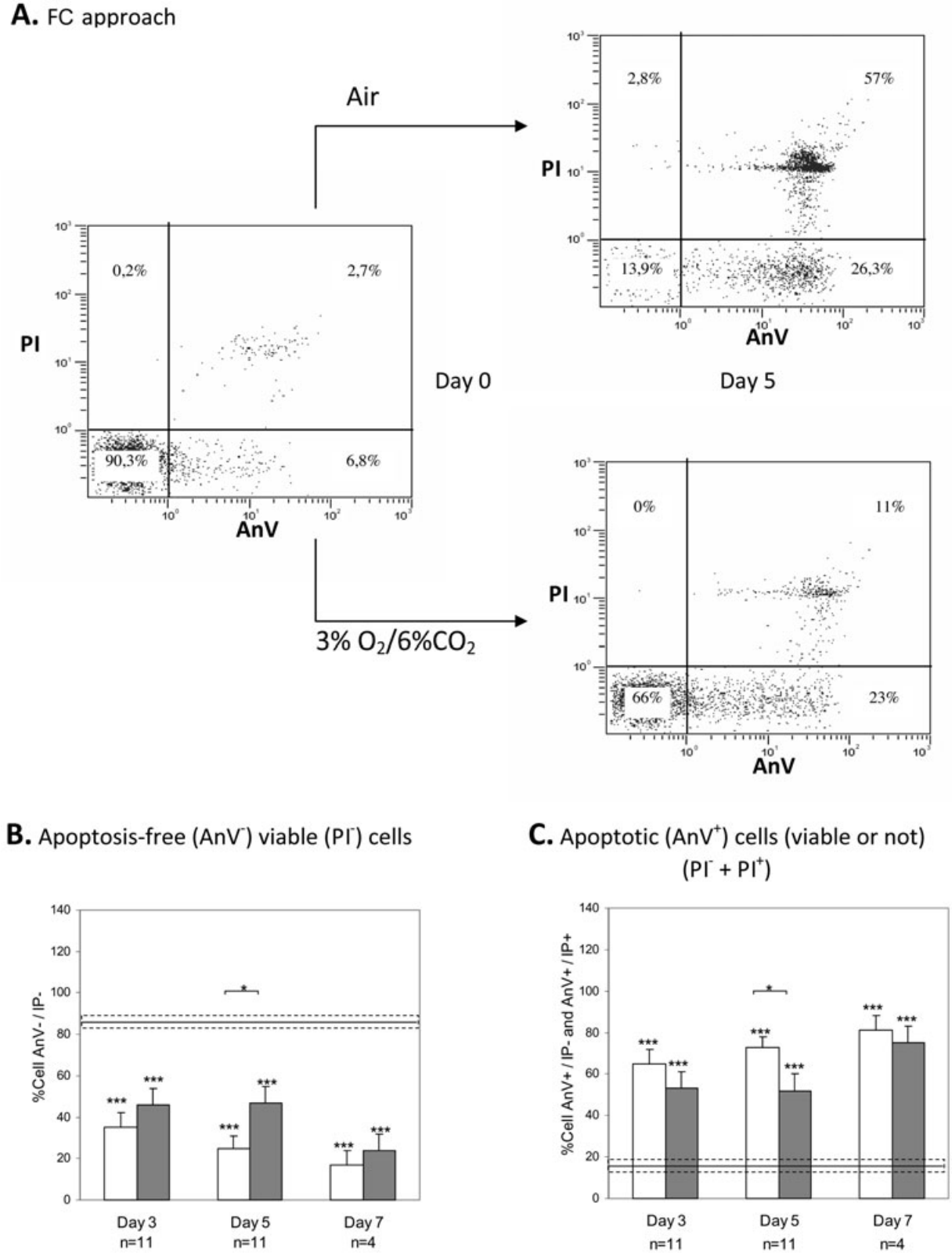

Fig. 2. Apoptosis in CD34+ cell population during the conservation in hypothermia in air (□) and at 3% O_2/6% CO_2 (■). As an example of analysis, the FC plots on Days 0 and 5 are presented from 1 of 11 experiments (A). Data are reported as mean ± SE (B,C); the differences between Day 0 and each experimental condition analyzed at the Day 3, 5, and 7 points are given in the form of asterisks on the top of the bars (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

and diluted whole blood.[20] Moreover, it is approaching the high-efficiency recovery of HCs after cryopreservation.[21]

We demonstrated that during 7 days storage, cells in G1 phase were five times better preserved at 3% O_2/6% CO_2 than in air. In experiments performed by Machaliński and colleagues,[7] the presence of air in storage vials improved CFC and CD34+ survival compared to the condition without air. However, the increasing amount of evidence for a role of intracellular ROS in hypothermic injury to diverse mammalian cells has been accumulated.[22] ROS are one of the main factors of apoptosis induction[23] in which the mitochondria have a major role.[24] The changes in mitochondrial membrane permeability could trigger the apoptosis cascade, while its stabilization prevents it. Since the pH values lower than 7 stabilize mitochondrial membrane permeability[25] and increased CO_2 concentration acidifies the storage medium (Table 1), one could propose that the atmosphere 3%O_2/6%CO_2 prevents apoptosis acting this way. The mechanism proposed seems to be plausible, since a similar phenomenon was evidenced during the liquid storage of

TABLE 1. Medium pH measurements*

	pH			
Atmosphere	Day 0	Day 3	Day 5	Day 7
Air (n = 4)	7.43 ± 0.07	7.39 ± 0.04	7.39 ± 0.09	7.38 ± 0.20
3%O_2/6% CO_2 (n = 4)	6.31 ± 0.02	6.44 ± 0.02	6.49 ± 0.01	6.56 ± 0.03

* The pH measures of cell suspension were made immediately after reaching the gas equilibrium (Day 0) and in the flask containing air or gas mixture (3%O_2/6% CO_2) conserved for 3, 5, and 7 days.

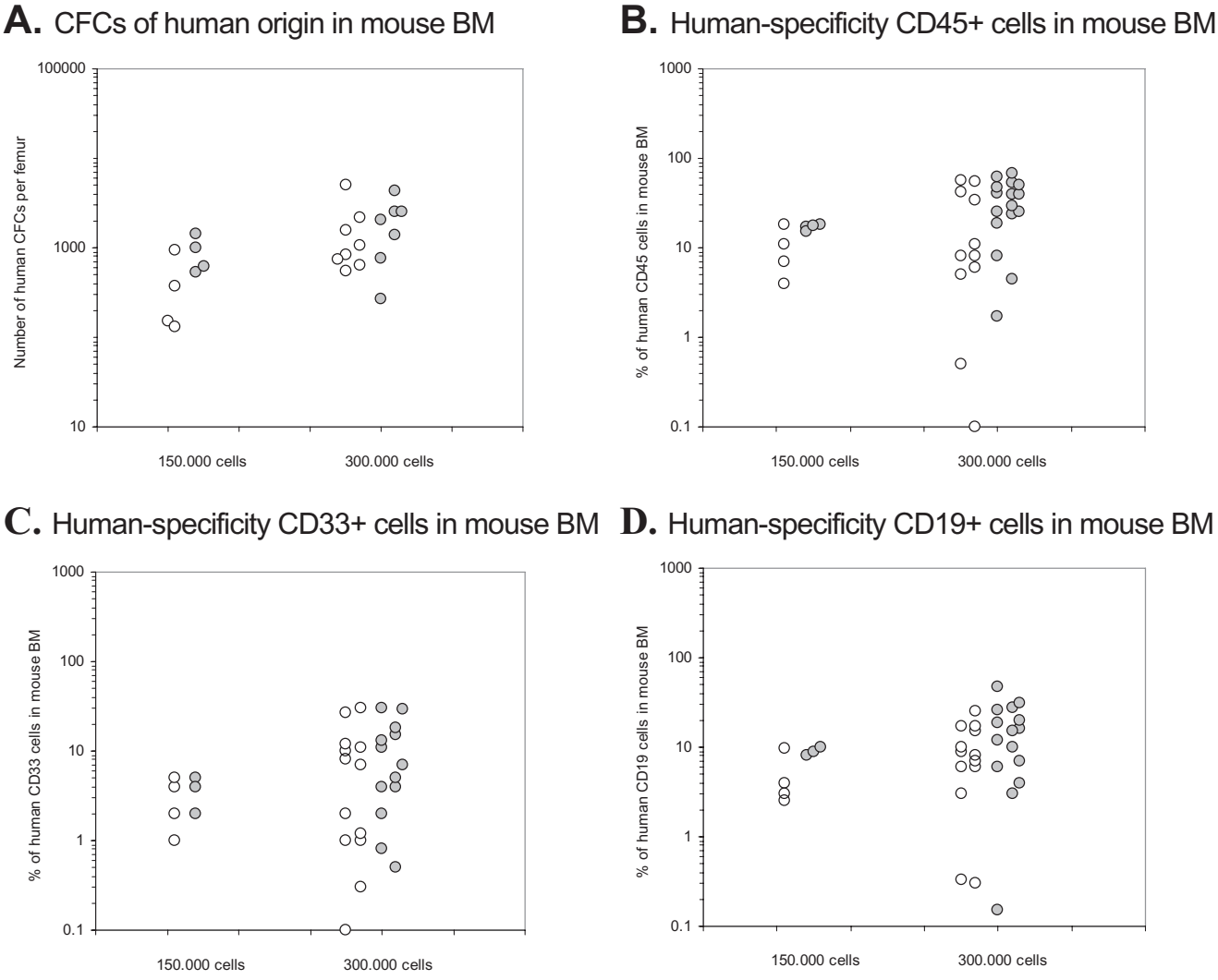

Fig. 3. Preservation of stem cells (SRCs) after 7 days of conservation in hypothermia in air (○) and at 3% O_2/6% CO_2 (●): human-origin CFCs (A), human antigen-specific CD45 (B), CD33 (C), and CD19 (D) chimerism in BM of NOG mice were studied 6 to 7 weeks after engraftment of Day 0 or Day 7 cells. Data are given as individual results cumulated from three separate experiments.

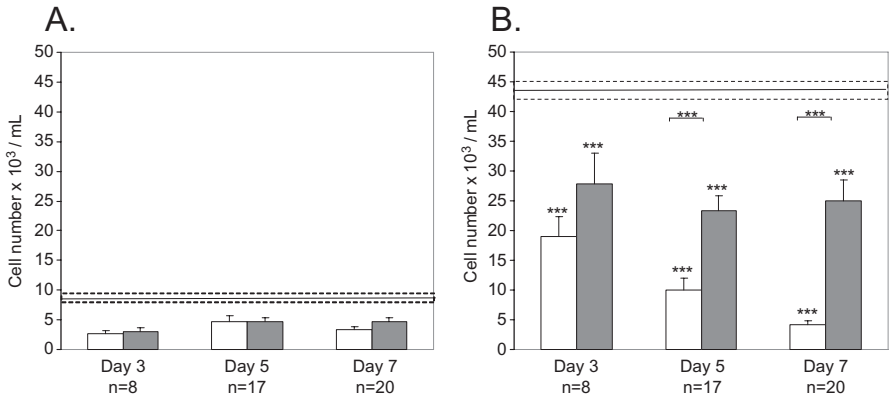

Fig. 4. Preservation of CD34+ cells in G0 (A) and G1 (B) phase of cell cycle in course of the conservation in hypothermia at air (□) and at 3% O_2/6% CO_2 (■). The nuclear fixation of PI was used to estimate the DNA content and the expression of the protein Ki-67 as a marker of active cell cycle (G1, SG2M). Data are reported as mean ± SE and the differences between Day 0 and specific condition analyzed are given in the form of stars on the top of the bars (*p < 0.05; **p < 0.01; ***p < 0.001).

chondrocytes: concentration of H^+ ions is positively correlated with the concentration of CO_2, and adjustment of medium pH with CO_2 is beneficial to stored cells.[26] At the same time, the mechanisms of positive or negative influence of pH and CO_2 on cell metabolism and survival could be independent. This phenomenon is demonstrated on different cell types including bacteria[27] and macrophages[16] and being confirmed in our ongoing studies on cord blood CD34+ cells in which the pH obtained with exposure of medium at gas mixture was mimicked by its adjustment by HCl: this enabled a fivefold lower preservation of CFC in comparison to gas mixture, although some other pH values, much lower than those in air-exposed flasks, achieved the gas mixture protection level (ongoing work—preliminary data).

The apoptosis of CD34+ cells stored in both gas conditions was induced partly by hypothermia, but we demonstrated that apoptotic events on CD34+ cells were more prominent in air-containing flasks. Therefore, the lower apoptosis in low oxygen storage conditions (3% O_2) could be related to a lower ROS production by CD34+ cells. Assuming that CD34+ cells exhibit some degree of metabolic activity even at 4°C (as suggested[7] for cord blood CD34+ cells judging from decrease of pH during storage), it could be hypothesized that a low O_2 concentration (3% in this case) is more adapted to a low level of aerobic/microaerofilic cell metabolism than atmosphere O_2 concentration (20%), i.e., hyperoxia or complete anoxia.

In fact, it is possible that cell preservation in our experiment was also improved by moderate hypercapnia (6% CO_2) decreasing oxygen consumption and sparing energy substrate during hypothermia,[13,28] thus decreasing ROS production independently of medium pH.[16] We also consider that an earlier suggestion that cytokines in storage media with serum are responsible for antiapoptotic signals on CD34+ cells in hypothermia[19] is not likely, since our defined storage medium was free of serum and cytokines. The concentrations of O_2 and CO_2 employed here, although based on a physiologic logic, were arbitrarily chosen and not experimentally optimized. The results of our ongoing experiments, aimed to explore this issue, are encouraging (ongoing work). The same reasoning is valid for the storage temperature, although the 4°C was a first-rate candidate for testing due to the practice and consequent logistic already existing.

Even if the degree of chimerism was in favor of better SRC preservation after 7 days at 3% O_2/6% CO_2 for the cell dose approaching a limiting dilution (150×10^3), the storage of CD34+ cells in both gas conditions resulted in a nonsignificantly different level of human chimerism after transplantation, indicating that the preservation of primitive stem cell activity was comparable in both gas conditions. The good preservation of stem cells in suspension exposed to air is in line with the literature data showing their high resistance and survival in different microenvironmental conditions, as in liquid storage systems at 4°C and room temperature,[6] during cryopreservation[3] as well as in normothermia in condition of ex vivo starvation.[29] Primitive stem cells are mostly in G0 phase of cell cycle.[30] In our study, both gas conditions preserved the majority of cells in G0, explaining the comparable "human" chimerism obtained after engrafting the CB CD34+ cells stored either in air or at 3% O_2/6% CO_2. Moreover, physiologically controlled hypometabolic state is associated with an elevation of antioxidant defenses.[31] Stem cells exhibit a high level of antioxidant defenses,[32] which could explain their high resistance during the storage in air. Altogether these data lead to the hypothesis that primitive stem cells, due to their metabolic peculiarities, are predisposed to survive different microenvironmental conditions developing state of controlled hypometabolism. These properties would be in line with our hypothesis considering that

eukaryotic stem cells reflect the primitive evolutionary stage of life (some anaerobic and aerofilic monocellular organisms).[33]

In conclusion, the introduction of low-oxygen and high-carbon-dioxide atmosphere in storage flasks had a highly positive effect on CD34+ cells in G1 phase of cell cycle. From a practical point of view, the simple introduction of desired gas mixture in defined media, and/or adjustment of pH during storage of HPCs and HSCs could be an efficient method to improve short-term (up to 7 days) conservation.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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