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# Cellular Properties of Human Erythrocytes Preserved in Saline–Adenine–Glucose–Mannitol in the Presence of L-Carnitine

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**L-Carnitine (LC) in the preservation medium during storage of red blood cells (RBC) can improve the mean 24-hr percent recovery in vivo and increase RBC life-span after reinfusion. The purpose of the study was to investigate the differences in the biochemical properties of RBCs stored in the presence or absence of LC, and the cell-age related responses to storage conditions and to LC. RBC concentrates in saline-adenine-glucose-mannitol (SAG-M) were stored in the presence or absence of 5 mM LC at 4°C for up to 8 weeks. RBC subpopulations of different densities were prepared by centrifugation on Stractan density gradient. Cells were sampled at 0, 3, 6, and 8 weeks, and hematological and cellular properties analyzed (MCV, MCHC, 4.1a/4.1b ratio as a cell age parameter, intracellular $Na^+$ and $K^+$). After 6 weeks, MCV of RBC stored in the presence of LC was lower than that of controls (6 weeks MCV: controls 95.4 ± 1.8 fl; LC 91.5 ± 2.0 fl; $n = 6$; $P < 0.005$). This was due to swelling of control cells, and affected mainly older RBCs. LC appeared to reduce or retard cell swelling. Among the osmotically active substances whose changes during storage could contribute to cell swelling, only intracellular $Na^+$ and $K^+$ differed between stored control RBCs and LC-treated cells. LC reduces the swelling of older cells during storage at 4°C in SAG-M, possibly by acting on the permeability of cell membrane to monovalent cations. Am. J. Hematol. 82:31–40, 2007.** © 2006 Wiley-Liss, Inc.

**Key words: RBC storage; L-carnitine; cell density; cell age; protein 4.1 a/b; monovalent cations**

## INTRODUCTION

The storage-related properties acquired by human erythrocytes preserved in solutions containing different additives have been widely characterized in the whole red blood cell (RBC) population. During storage, a progressive deterioration in the cellular, membranous, and metabolic properties of erythrocytes have been shown to take place, resulting in cell swelling, hemolysis, loss of deformability, and shape changes [1,2]. Shedding of membrane fragments as vesicles [3], and the inhibition in the cold of the $Na^+/K^+$-ATPase, with the consequent increase in intracellular $Na^+$ and decrease in intracellular $K^+$ contents [4], also occur. The concentrations of metabolites such as glucose, 2,3-BPG, and ATP decline with storage, and lactate accumulates, determining a pH decrease from 7.0 to ~6.5 after 6 weeks of storage [5,6].



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Since the discovery that the ratio of protein 4.1a to protein 4.1b, which are a part of the red cell membrane cytoskeleton, is correlated with erythrocyte life span in mammals [7–9], several authors have adopted the 4.1a/4.1b ratio as a parameter to characterize the absolute age of density-fractionated human erythrocytes [10–12]. Results obtained so far have shown that the population of circulating red cells is in fact very heterogeneous, with respect to membrane surface, cell volume, cation and water content, and cell deformability [12,13]. It is thus reasonable to suspect that subpopulations of erythrocytes of different age will acquire different cellular properties when faced with the storage conditions, and behave differently after reinfusion [14].

The presence of L-carnitine (LC) in the preservation medium during erythrocyte storage has been shown to improve the mean 24-hr percentage of cell recovery in vivo and to enhance the life-span of stored red cells after reinfusion [15]. The beneficial action of LC on stored RBCs may be related to biophysical and/or metabolic interventions on RBC membrane. LC mainly acts by transporting long-chain fatty acids to the sites of β-oxidation in the mitochondrial matrix [16]. Despite the lack of mitochondria, evidence for a role of LC in red cell metabolism is suggested by the presence of LC and carnitine palmitoyl-transferase in the red cell, and their involvement in membrane phospholipid fatty acid turnover [17–19]. Furthermore, LC and its short chain esters were found to favorably affect the biophysical status and rheological properties of erythrocyte membrane under normal and adverse conditions [20–23].

The purpose of this study was to gain further information on the effect of LC on human red cells storage by applying more sophisticated biochemical studies regarding the recovery and properties of erythrocytes of different age.

## DESIGN AND METHODS

### Donation and Processing of Blood

**Preparation of storage units.** Whole blood (450 ± 50 ml) was collected from healthy volunteer donors into quadruple blood bags (Terumo, Tokyo, Japan) containing 63 ml of citrate-phosphate-dextrose as an anticoagulant in the primary bag and 100 ml of SAG-M, as a red cell additive solution in a satellite bag. In some experiments, AS3 (Nutricell, Pall Medical, Covina, CA) was used as an additive. Units were centrifuged at 5,375*g* for 6 min at 22°C in a Sorvall RC-3C Plus centrifuge equipped with a rotor H6000A/HBB6 (DuPont, Wilmington, DE) between 2 and 4 hr of collection. The plasma was expressed into a satellite bag and stored at −40°C. Packed red cells were then resuspended in SAG-M to final Hct of (60 ± 0.5)% and filtered through a high efficiency leukodepletion filter (PALL BPF4BE, Pall Medical Corporation).

For each experiment, two filtered SAG-M red cell units, obtained from donors of the same age range (23–41 years) and ABO group, were pooled in a NTR1000 transfer bag (NIPRO, Tokyo, Japan) using a sterile connection device (SCD312, Terumo), mixed, then divided into two equal weight subunits (A and B) using NTR600 transfer bags (NIPRO), and stored at 2–6°C in a refrigerator. The weight was recorded on each bag to calculate the volume [volume (ml) = weight (g)/1.055].

**Addition of L-carnitine.** On the day following collection, after ~20 hr storage at +4°C, 10 ml of 0.15 M LC, to achieve a final supernatant concentration of 5 mM, or an equal volume of saline solution were added to A and B subunits, respectively, under filter-sterile conditions using a site coupler (Medication Injection Plug for Blood Bag, Terumo). The subunits were stored at 2–6°C for up to 8 weeks. Aliquots of 60 ml were aseptically collected from each subunit after 30 min (time 0), 3, 6, and 8 weeks of storage for the determination of the following parameters: hemoglobin (Hb) concentration, red cell count and indices, erythrocyte morphology and osmotic fragility, percent hemolysis, free Hb, and pH. The concentrations of adenosine triphosphate (ATP), 2,3-bisphosphoglycerate (2,3-BPG), lactate, and glucose were also measured. Cell-free supernatants from the storage units were analyzed for cell membrane vesicle content. Red cells were finally processed for monovalent cation assay, ghost membrane preparation (for gel electrophoresis and 4.1a/4.1b quantification), and for density separation on polyarabinogalactan (Stractan) gradients.

### Density Separation

**Discontinuous gradient preparation.** The method of Corash et al. [24] was adopted with modifications (see Ref. 25). Discontinuous gradients were made by layering 6 polyarabinogalactan solutions of different densities (1.075, 1.090, 1.095, 1.098, 1.101, and 1.103 g/ml) in 18 ml tubes (16 × 102 $mm^2$ Beckman Ultraclear, Beckman Instruments, Palo Alto, CA). A high-density cushion was first layered at the bottom of each tube using a solution of 1.137 g/ml polyarabinogalactan.

**Separation of erythrocytes according to density.** Cell suspension (40 ml) collected from the storage unit were centrifuged at 1500*g* for 5 min. Superna-

tants were saved and the packed cells resuspended with fresh SAG-M to a Hct of 80%. This suspension was layered on the density gradients (2.5 ml cell suspension/tube). Tubes were centrifuged at 20°C for 20 min at 74,000*g* ($r_{av}$) in a swinging arm rotor (SW28, Beckman Instruments) in an ultracentrifuge (L-65, Beckman Instruments). From each tube, six density fractions were collected. Fractions of corresponding densities from four tubes were pooled and cells washed three times with phosphate buffered saline (154 mM NaCl, 4.5 mM KCl, 5 mM phosphate, 1 mM glucose, pH 7.4, 300–310 mosm/kg $H_2O$). A 50% Hct suspension in PBS was prepared from each subpopulation of cells and its volume precisely determined with a syringe, to calculate the recovery of cells in each fraction. After centrifugation and removal of the supernatant, cells were resuspended in SAG–M at 20% Hct. From this suspension, samples were taken for cell count and hematological analysis, for determination of $Na_i^+$ and $K_i^+$, and for ghost membrane preparation.

**Incubation of stored cells before density-separation.** Erythrocytes stored at 4°C perform glycolysis at a rate about 1/50 of that at 37°C [26]. Lactate produced inside the cells equilibrates with the external medium and the overall osmolality of the aqueous compartments increases (2 mol of lactate are formed per mole of glucose consumed). When 42 days stored erythrocytes are transferred from the storage unit to an isotonic medium, a water influx rapidly ensues, because of the relative hyper-osmolality of the cytoplasm, at least in part attributable to lactate. At the same time, lactate efflux also begins as this metabolite is transported into the medium. To study the impact of lactate on the density properties of stored erythrocytes, in some experiments cells from the storage units were density separated before and after 1 hr incubation at room temperature in 20 volumes of PBS [27]. The release of lactate in the extracellular medium during this period was monitored by sampling at 5 and 60 min of incubation. Cells were then sedimented and resuspended to 80% Hct with SAG-M and loaded on discontinuous gradients for density separation as described above.

**Hematological and biochemical procedures.** Blood cell count, Hct, Hb level, and erythrocyte indices were obtained with a Sysmex NE-8000 automated hematology analyzer (TOA Medical Electronics, Kobe, Japan). Routine hematological investigations were performed as previously described [28]. Free extracellular Hb was determined by a spectrophotometric scanning technique [29]. The concentration of ATP, 2,3-BPG, lactate, and glucose was measured according to Beutler [30]. pH was determined by hemogasanalyser IL1620 (Instrumentation Laboratory, Lexington, MA).

**Ghost membrane preparation and gel electrophoresis.** Purified ghost membranes were prepared from erythrocytes (total population or subpopulations of different density), by hypotonic lysis at 4°C, according to the method of Marchesi and Palade [31]. All buffers contained 0.2 mM of the antiprotease phenyl-methyl-sulfonyl-fluoride (PMSF). For sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of membrane proteins, one volume of ghost suspension was treated with 0.5 volumes of denaturing buffer (50 mM Tris(hydroxymethyl) aminomethane (Tris)/HCl, 5 mM EDTA, 5% SDS, 35% sucrose, 200 mM DTT, pH 6.8) and incubated for 15 min at 60°C. Triplicates of each sample were run in a gel (7% acrylamide, gel dimensions 140 × 130 × 1.5 $mm^3$) according to Laemmli [32]. Gels were stained with Coomassie Blue.

**Quantification of the ratio protein 4.1a/protein 4.1b.** The ratio between membrane protein 4.1a and 4.1b was calculated after densitometric quantification of the protein bands in the gel as specified elsewhere [25]. When 4.1a/4.1b ratio was evaluated in subpopulations of density separated red cells, a "normalized 4.1a/4.1b" value was calculated, defined as the ratio between the 4.1a/4.1b value of a given subpopulation of cells and the 4.1a/4.1b value of the total population of cells from which the subpopulation was obtained by density separation. This procedure reduced experimental variability in the determination of the cell-age parameter.

**Quantification of membrane vesiculation.** Cell free supernatants from 42 days stored red cell concentrates, and the supernatants from two subsequent washes of the cells with PBS were pooled and processed for membrane vesicle quantification as follows. Microvesicles and membrane fragments were sedimented at 27,000*g* at 20°C for 30 min. The sediment was resuspended with a suitable volume of 150 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.4 and used as such for "raw vesicles" quantification, or further processed for purification of microvesicles on self forming gradients of Percoll (Pharmacia Biotech, Uppsala, Sweden) [33]. Microvesicles (either "raw" or "purified") were quantified as total phospholipid phosphorus.

**Other assays.** Proteins were assayed according to the method of Lowry [34]. Lipids were extracted from microvesicles as described by Rose and Oaklander [35]. Phospholipids were quantified as inorganic phosphate (Pi) after extraction from membranes and acid digestion, according to Bartlett [36]. Intracellular monovalent cations ($Na^+$ and $K^+$) were quantified by atomic-emission flame photometry

with an atomic absorption spectrometer (Mod. 1100B, Perkin Elmer Corp., Norwalk, CT), on a dilution of cell lysates. Duplicate aliquots of erythrocytes were washed three times at 4°C with a nominally $Na^+/K^+$-free buffer of the following composition: 100 mM sucrose, 10 mM glucose, 65 mM $MgCl_2$, 10 mM Tris/3-(*N*-morpholino) propanesulfonic acid (MOPS), pH 7.4, 305–315 mosm/kg $H_2O$ ($[Na^+] < 10$ μM, $[K^+] < 20$ μM). Cells were then resuspended in the same buffer at approx. 20% Hct and the same suspension used for parallel determination of Hb content, microhematocrit, monovalent cations.

**Statistics.** Statistical analysis was performed using paired *t* test. Differences were considered significant at $P \leq 0.05$.

## RESULTS

### Cellular Properties of the Total Population of Erythrocytes Stored in SAG-M

A detailed panel of hematological and biochemical parameters, evaluated on the total population of erythrocytes, is given in Table I. In control samples, mean cell volume (MCV) increased during storage, an average 6% increase at week 6, and mean corpuscular Hb concentration (MCHC) correspondingly decreased. A statistical significant difference was observed for both MCV and MCHC between control and LC-treated samples at week 3 ($P < 0.005$), week 6 ($P < 0.005$), and week 8 ($P < 0.05$). In a number of experiments, total cell volume was also measured by a microhematocrit method (not shown), leaving the cells in the original storage medium, thus avoiding possible artifacts due to sudden mixing with exogenous buffers, which could occur when cells are processed with automatic analyzers. With both methods, data confirmed that swelling of control cells had occurred during storage, and that LC significantly prevented or retarded the increase in cell volume.

Table I also shows statistical significant differences between control and LC-treated samples concerning the intracellular monovalent cations: $Na_i^+$ at week 6 was approximately three times the value at time zero ($P < 0.005$), while $K_i^+$ at the same time was about 60% of the original ($P < 0.05$). Importantly, control samples displayed a significantly higher content of both $Na^+$ and $K^+$ than LC samples at week 6 (Table I). Cell volume is determined by intracellular water content, which for normal fresh erythrocytes is regulated mainly by $K_i^+$ and $Na_i^+$, as the principal osmolytes [37]. Thus, the differences in $Na_i^+$ and $K_i^+$ between samples stored in the presence and in the absence of carnitine suggested a possible relation of cell swelling to monovalent cation contents.

### Cellular Properties of Subpopulations of Erythrocytes of Different Density During Storage, as a Function of Cell Age

During storage of erythrocytes for up to 8 weeks in SAG-M, with or without LC, changes in cell density occurred (see Fig. 1). The average cell recovery (bars), and the mean cell age parameter (4.1a/4.1b, lines) were measured in six independent experiments, for each of the six subpopulations of different density prepared at various time point during storage. At time zero, the density distribution and the 4.1a/4.1b ratio profiles were similar in control and LC samples (see Fig. 1), and the 4.1a/4.1b values increased with the density of the cell population, as expected from a typical distribution of erythrocytes of different age [12].

With the progression of storage, the recovery of low-density cells increased, an effect that was more pronounced in controls than in LC-treated samples. This resulted in a significantly higher recovery of cells in the low-density subpopulation (F1) of controls when compared with LC F1 subpopulation ($P < 0.05$), already after 3 weeks of storage. The 4.1a/4.1b values in the F1 subfraction were higher for control samples than for LC samples, indicating that a population of cells that were middle-aged/senescent at the beginning of storage was mostly affected by swelling, since it was progressively recovered in lower density regions of the gradient. Correspondingly, a decrease in cell recovery in subpopulations denser than F2 was observed, an event that mirrored the increased recovery of F1 cells, and that was more pronounced for control than for LC samples.

On the whole, as shown in Fig. 1 (week 8 panel, controls), the oldest erythrocytes of the original population of cells, those carrying the higher 4.1a/4.1b values, underwent a density decrease that caused them to migrate in fractions F1 and F2, increasing the average 4.1a/4.1b values detected in these two subpopulations. For LC-treated samples, the decrease in density of old erythrocytes was less significant than in controls, and caused these cells to shift only to the F2–F3 regions of the gradient.

### Effect of Intracellular Lactate on the Storage Induced Cell Density Changes

To determine the role of lactate accumulated during storage in affecting cell density when cells were transferred from the storage units to the density gra-

**TABLE I. Properties of Erythrocytes Stored at 4°C in SAG-M**

| | $t = 0$ | | Week 3 | | Week 6 | | Week 8 | |
|---|---|---|---|---|---|---|---|---|
| | Control | L-Carnitine | Control | L-Carnitine | Control | L-Carnitine | Control | L-Carnitine |
| MCV (fl) | 89.9 ± 3.3 | 89.8 ± 3.1 | 93.6 ± 3.1 | 91.4 ± 2.8** | 95.4 ± 1.8 | 91.5 ± 2.0** | 97.0 ± 4.0 (3) | 93.5 ± 2.5 (3)* |
| MCHC (g/dl) | 33.3 ± 0.9 | 33.3 ± 0.9 | 32.3 ± 0.4 | 33.0 ± 0.5** | 31.4 ± 0.4 | 32.8 ± 0.5** | 31.1 ± 1.0 (3) | 32.3 ± 0.6 (3)* |
| Free Hb (mg/dl) | 50.3 ± 10.7 | 49.7 ± 16.1 | 89.5 ± 20.5 | 69.7 ± 15.1 | 133.9 ± 51.2 | 109.8 ± 31.7 | 282.1 ± 96.4 (3) | 235.5 ± 87.1 (3) |
| Hemolysis (%) | 0.17 ± 0.07 | 0.18 ± 0.08 | 0.22 ± 0.09 | 0.21 ± 0.13 | 0.36 ± 0.14 | 0.32 ± 0.13 | 0.83 ± 0.05 (3) | 0.71 ± 0.23 (3) |
| Glucose (mM)[a] | 22.3 ± 1.5 (3) | 24.1 ± 4.2 (3) | 15.6 ± 4.6 (3) | 13.9 ± 5.2 (3) | 10.5 ± 1.1 (3) | 9.9 ± 2.2 (3) | | |
| Lactate (mM)[a] | 4.1 ± 2.7 | 3.6 ± 1.4 | 22.3 ± 8.5 | 22.0 ± 6.6 | 29.6 ± 3.8 | 29.9 ± 4.8 | 36.7 ± 5.1 (3) | 34.7 ± 2.1 (3) |
| 2,3-BPG (μmol/g Hb) | 10.09 ± 2.00 | 10.20 ± 1.59 | 0.82 ± 0.70 | 0.98 ± 0.86 | 0.65 ± 0.58 | 0.61 ± 0.52 | 0.32 ± 0.48 (3) | 0.29 ± 0.25 (3) |
| ATP (μmol/g Hb) | 4.10 ± 0.47 | 3.52 ± 0.95 | 3.46 ± 0.67 | 3.69 ± 0.75 | 2.66 ± 0.56 | 2.60 ± 1.06 | 1.24 ± 0.04 (3) | 1.03 ± 0.10 (3) |
| pH | 7.01 ± 0.05 | 7.00 ± 0.05 | 6.61 ± 0.05 | 6.60 ± 0.05 | 6.42 ± 0.05 | 6.41 ± 0.05 | 6.38 ± 0.06 (3) | 6.37 ± 0.07 (3) |
| 4.1a/4.1b | 1.880 ± 0.298 | 1.892 ± 0.272 | 1.779 ± 0.178 | 1.785 ± 0.116 | 1.908 ± 0.213 | 1.896 ± 0.199 | 1.938 ± 0.213 (3) | 1.936 ± 0.169 (3) |
| $Na_i^+$ (μEq/g Hb)[b] | 34.1 ± 16.9 (4) | 33.3 ± 15.8 (4) | 82.9 ± 4.2 (4) | 77.0 ± 6.0 (4)* | 113.9 ± 11.1 (5) | 105.9 ± 9.1 (5)** | | |
| $K_i^+$ (μEq/g Hb)[b] | 258.1 ± 23.1 (4) | 255.9 ± 33.7 (4) | 198.7 ± 11.4 (4) | 188.3 ± 13.1 (4)* | 168.8 ± 9.0 (5) | 158.2 ± 7.6 (5)* | | |
| $Na_i^+ + K_i^+$ (μEq/g Hb)[b] | 292.2 ± 10.3 (4) | 289.2 ± 22.1 (4) | 281.7 ± 10.6 (4) | 265.4 ± 13.7 (4)* | 282.7 ± 15.2 (5) | 264.1 ± 15.0 (5)* | | |
| Raw vesic. (nmol Pi/ml RBC) | | | | | 29.83 ± 7.93 (3) | 31.73 ± 9.46 (3) | | |
| Purif. vesic. (nmol Pi/ml RBC) | | | | | 14.70 ± 7.82 (3) | 16.33 ± 6.23 (3) | | |

$N = 6$ unless otherwise indicated by the number in brackets. Results are expressed as mean ± standard deviation.

This table is focused on the statistical comparison between control and LC-treated samples. When, within a given sample, the changes over time for some parameters are obvious (e.g., decrease in 2,3-BPG or ATP concentrations), their statistical evaluation has been omitted for sake of clarity; it will however be discussed whenever the differences deserve a comment.

[a]Concentration in the whole cell suspension from the storage unit.

[b]Cations were assayed in 3 of the 6 main experiments and in 1 (or 2) additional independent samples.

*$P < 0.05$.

**$P < 0.005$ paired $t$ test between control and L-carnitine samples.

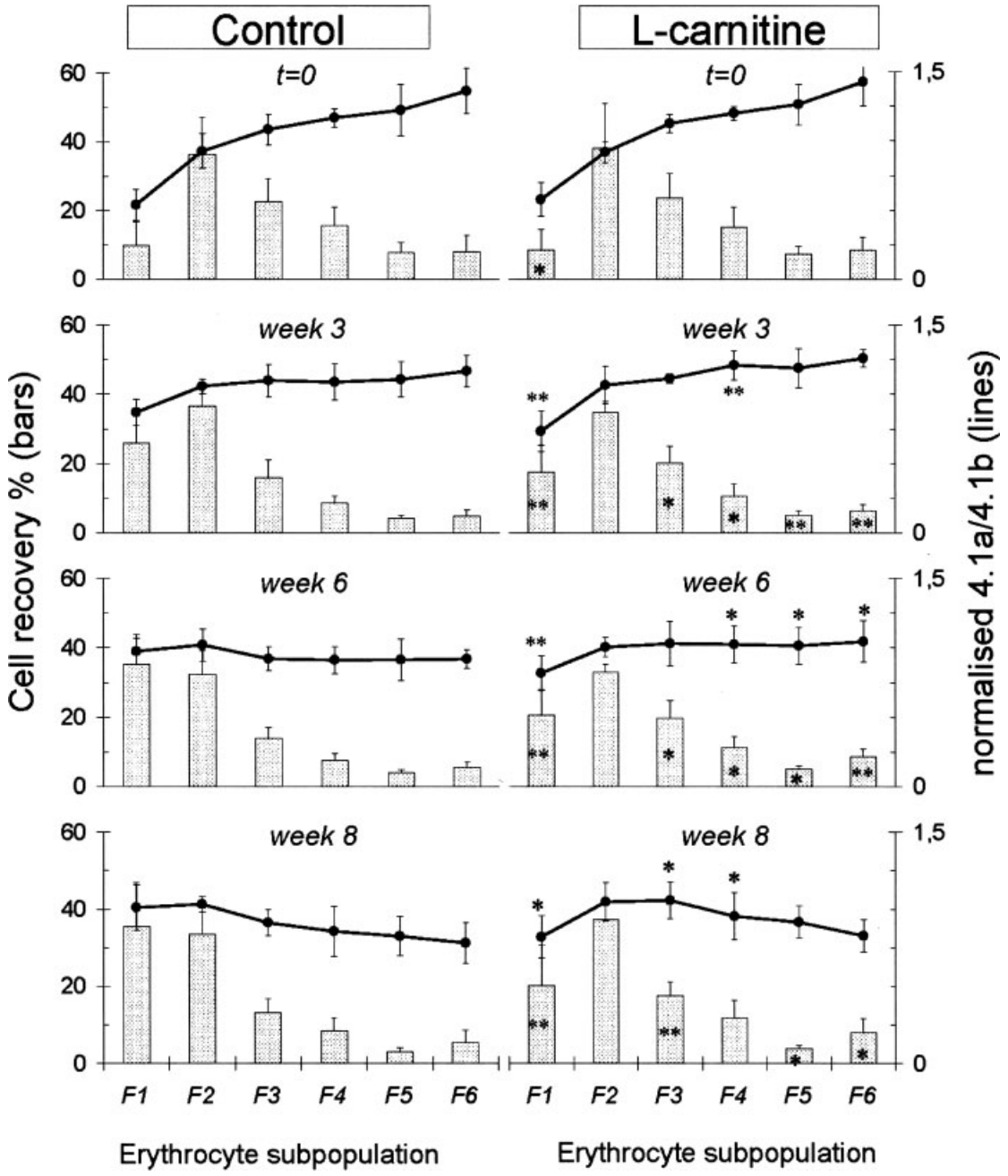


**Fig. 1. Separation of erythrocytes stored for various time intervals at 4°C in SAG-M in the absence (control, left) and presence (right) of LC, on discontinuous density gradients of Stractan. Quantitative presentation of cell recovery (BARS), and of the age parameter, 4.1a/4.1b ratio (LINES), measured in each subpopulation of cells of increasing density from F1 to F6. Data represent the mean of six ($t = 0$, week 3, week 6), or three (week 8) independent experiments. For a definition of "Normalized 4.1a/4.1b", see "Materials and Methods" section. Error bars represent standard deviations. Asterisks indicate the statistical significance of the difference between "control" and "LC" samples: * $P \leq 0.05$; ** $P \leq 0.005$.**

dient, and the relative importance of this phenomenon in control and LC-treated samples, aliquots of 42 days stored red cells were density fractionated immediately after the sampling from the storage units, and after 1 hr incubation in 20 volumes of PBS at room temperature, to allow the efflux of intracellular lactate [27]. As shown in Fig. 2, this maneuver resulted in a decrease in cell volume of swollen cells, indicating that the factors that determined cell swelling were partially reverted by suspending

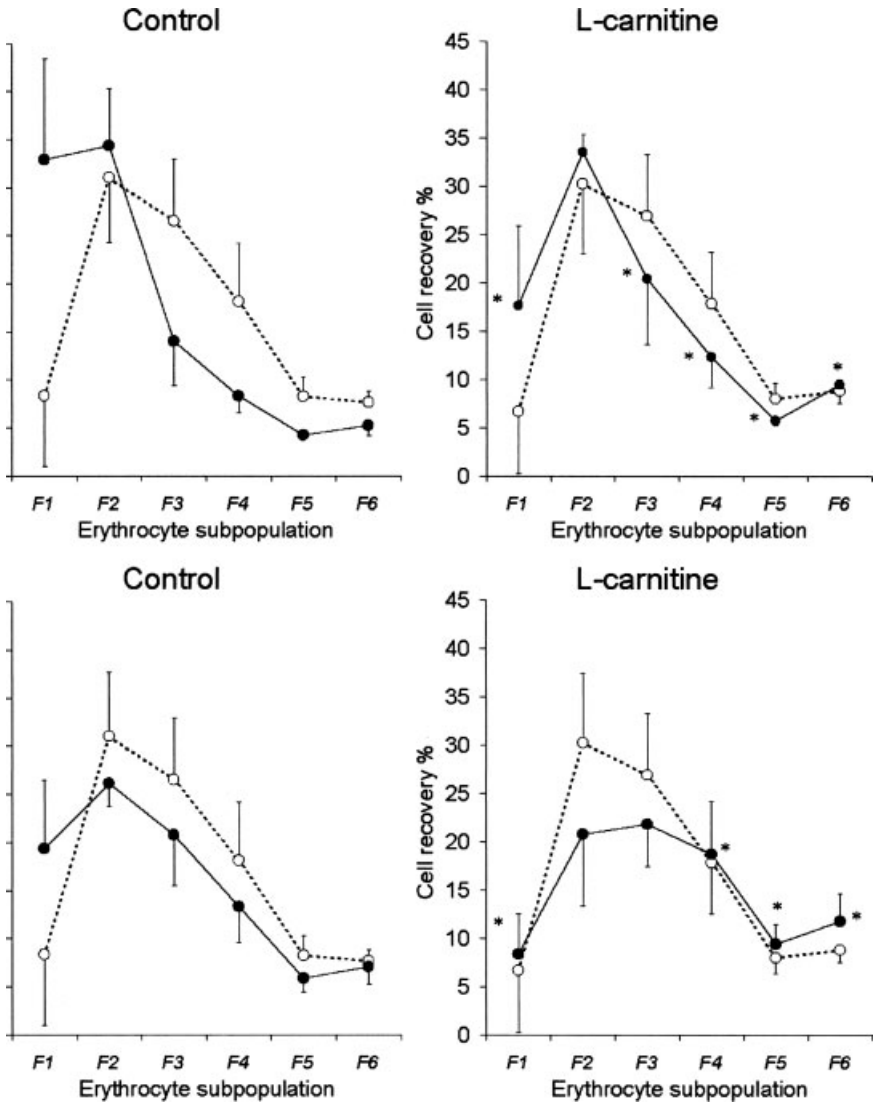


**Fig. 2. Results of three experiments in which erythrocytes stored for 6 weeks were density-separated before (top panels) or after (bottom panels) preincubation with 20 volumes of PBS for 1 h at room temperature. Dashed lines represent the density distribution profiles of cells at time zero. Error bars represent standard deviations. Asterisks indicate the statistical significance of the difference between control and LC samples: * $P \leq 0.05$.**

the cells in a large volume of isotonic buffer. However, the density distribution profiles were not restored to those observed at time zero, and the swelling of control cells relative to LC-treated cells was still clearly detectable.

This indicated that (a) the swelling of cells as detected by density-separation was probably amplified, because of the incomplete washing of the accumulated lactate before loading in the gradients; (b) the residual difference in the density distribution profiles between control and LC samples was consistent with an increased amount of slowly diffusible, intracellular osmolytes, other than lactate, in the former compared to the latter. Lactate efflux occurred in fact at a comparable rate in control and LC-treated samples (not shown).

### $Na^+$ and $K^+$ Content in Subpopulations of Erythrocytes of Different Density During Storage

Intracellular monovalent cations were analyzed in all subpopulations of erythrocytes density-separated at time zero, week 3, and week 6. The results are presented in Fig. 3 together with the percent recovery of cells (bars) and the 4.1a/4.1b ratios (top panels) in each subpopulation. At time zero, cells of increasing density and age are characterized by decreasing $K^+$ content and slightly increasing $Na^+$

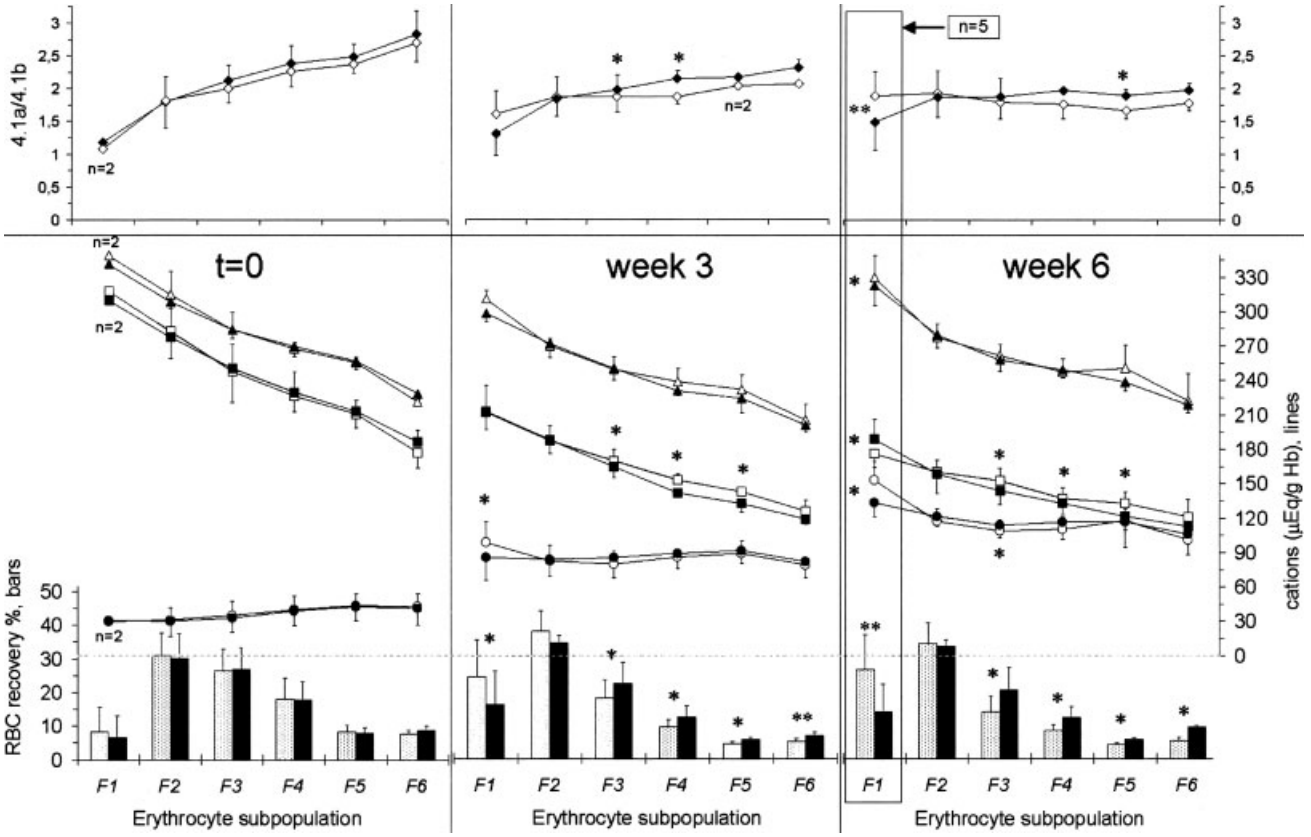


**Fig. 3. Bottom panels: cell recovery (bars) and intracellular monovalent cations (lines: $Na^+$, circles, $K^+$, squares and $Na^+$+ $K^+$, triangles) in subpopulations of erythrocytes of different density obtained at time zero, week 3, and week 6 of storage in the absence (white symbols) or presence (black symbols) of LC. Top panels: 4.1a/4.1b ratios of erythrocyte subpopulations. Results were obtained in three independent experiments, except where otherwise indicated. Error bars represent standard deviations. Asterisks indicate the statistical significance of the difference between "control" and "LC" samples: * $P \leq 0.05$; ** $P \leq 0.005$.**

content per gram Hb, consistent with the decrease in cell size and water, at constant Hb content, which take place during physiological erythrocyte senescence. No differences were observed between control and LC samples. At week 3 and 6 of storage, all subpopulations of different density displayed an increase in $Na^+$ and decrease in $K^+$. In particular, the subpopulation F1 displayed the highest increase in $Na^+$ content. Taken together with the observation that cell recovery in F1 also increased during storage, this indicated that at week 6 the light fraction of the gradient became populated by cells that swelled, at least in part, because of the relative accumulation of total cations, in particular sodium. The increased 4.1a/4.1b ratio in cells of F1 at week 6 suggested that older erythrocytes were mostly affected by cell swelling. In LC treated samples, the described modifications took place to a lesser extent. Thus, LC appeared to prevent, or retard, the swelling of older cells that resulted, for untreated cells, in the accumulation of a large population of light erythrocytes characterized by high $Na^+$ content and relatively older age.

## DISCUSSION

In this study we have reported on the effect of LC in partially preventing, or retarding, the cell swelling that affects human erythrocytes during storage under blood bank conditions.

We exploited a combination of density-separation of erythrocytes at various stages of storage, and of quantification of 4.1a/4.1b ratios to determine whether, during storage, swelling uniformly affected all cells or particular subpopulations of erythrocytes. In addition, since the 4.1a/4.1b parameter of the total population of cells did not change over 8 weeks of storage (Table I), no major selection (e.g., via hemolysis) of a population of cells occurred during storage. Importantly, LC did not interfere with the chemical properties of protein 4.1, as no major differences

between control and LC-treated samples were detected in the 4.1a/4.1b values of the total population of cells at various time points (see Table I).

We indeed observed that a fraction of relatively older cells underwent a larger density decrease, as the progressive increase in the 4.1a/4.1b values in the lower density regions of the gradient indicated. This phenomenon was more pronounced in controls than in LC-treated samples. The data also suggested that another, albeit less pronounced, process might have occurred, affecting in this case a subset of younger cells that appeared to shrink during storage. In fact, despite a relative constancy in the recovery of high density cells (Fig. 1, fractions F4–F6), their 4.1a/4.1b values decreased during storage, suggesting that the population of senescent erythrocytes was "diluted" by younger cells that became dehydrated.

Since the developing of differences in cell volume between control and LC-treated erythrocytes was the most significant and reproducible event taking place during storage, we focused on possible explanations for this phenomenon.

We have shown that the intracellular accumulation of lactate is an important factor in determining cell swelling, as is evident when stored erythrocytes are rapidly transferred to a lactate-free isotonic medium. However, we still detected a higher cell swelling in control, 6-weeks stored erythrocytes, with respect to both time-zero control samples and 6-weeks stored LC samples, after cells had been washed with fresh isotonic medium to remove intracellular lactate. Moreover, no significant differences developed, between control and LC samples, during storage either in the amount of lactate produced or in the amount of 2,3-BPG lost. This suggested that lactate accumulation, and the increase in intracellular $Cl^-$ as a replacement for the 2,3-BPG lost [27], could have contributed to the swelling of control and LC samples to the same extent, but that other factors were responsible for the additional swelling that affected control cells. When looking at the $Na_i^+$ and $K_i^+$ content of the unseparated total population of stored cells (see Table I), it appeared that LC treatment induced higher loss of total cations than in controls, and therefore, shrinkage of LC-treated cells relative to untreated cells would be expected during storage. In fact, cell volume did not appear to change in LC-treated samples, contrary to control samples where cell volume increased.

Analysis of intracellular cations in all subpopulations of cells at each time point during storage reveals a complex situation, whereby younger cells lose potassium and older cells gain sodium. The higher content of monovalent cations in the total population of cells in controls relative to LC-treated samples could be due to the relative increase in the recovery of swollen cells, with high $Na^+$ content, in the former, compared to the latter. It is also likely that in LC-treated samples a slightly higher loss of potassium and the shrinkage of a larger amount of younger cells might have taken place relative to controls. However, from the measurements of 4.1a/4.1b ratios, we tentatively deduced that this latter phenomenon was of lesser importance when compared with the increase in the amount of swollen cells, with high sodium content in the F1 subpopulation of controls with respect to LC-treated samples.

In the present study, we have not evaluated the survival of erythrocytes after reinfusion; therefore, it is not possible to set a direct link between the better performance after reinfusion of red cells stored in the presence of LC, as previously described [15].

However, LC appeared to benefit older cells that would otherwise accumulate abnormal amounts of $Na^+$, and osmotically swell. On the other hand, the shrinkage that a subset of younger cells underwent during storage in the presence of LC could limit the extent of the regulatory volume decrease, and therefore the $K^+$ loss through $K^+$-$Cl^-$ cotransporter that these cells would otherwise activate when transfused [38], thus ameliorating their survival potential.

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