Addition of ascorbic acid solution to stored murine red blood cells increases posttransfusion recovery and decreases microparticles and alloimmunization

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BACKGROUND: The storage of red blood cells (RBCs) results in numerous changes, which over time result in decreased recovery of transfused RBCs. In addition (at least in animal models), stored RBCs can be more immunogenic and also stimulate the systemic release of inflammatory cytokines in transfusion recipients. One component of the RBC storage lesion is the accumulation of oxidative damage. We tested the hypothesis that adding a chemical antioxidant (ascorbic acid) to stored RBCs would improve the quality of the stored RBCs.

STUDY DESIGN AND METHODS: RBCs were harvested from FVB.HOD mice that express an RBC-specific model transgene (HOD) and stored for 14 days with either ascorbic acid in saline or saline alone. Twenty-four-hour posttransfusion recovery of RBCs was tracked by flow cytometry. Alloimmunization was monitored by flow cytometry crossmatch. Cytokines were monitored by multiplex bead arrays.

RESULTS: RBCs stored under standard conditions had decreased 24-hour posttransfusion recovery and increased induction of both alloantibodies and interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 secretion in the mouse recipients. Addition of ascorbic acid from 3.6 to 10.8 mmol/L resulted in a significant decrease in microparticle formation, an improved RBC 24-hour posttransfusion recovery ($p < 0.01$), and a decrease in recipient alloimmunization ($p = 0.0001$). Induction of MCP-1 and IL-6 secretion was not decreased by ascorbic acid.

CONCLUSIONS: These data indicate that the addition of ascorbic acid solution to RBCs during storage has a beneficial effect on recovery and immunogenicity of RBCs, but not cytokine induction. The addition of ascorbic acid (or other antioxidants) to human RBCs may have beneficial effects.

The ability to store donated units of red blood cells (RBCs) was a seminal achievement in the mid-1900s and resulted in the ability to utilize RBC transfusion as a supportive therapy in surgery and cancer patients. Although simple in composition, solutions based on citrate and dextrose continue to be the basis for the Food and Drug Administration (FDA)-approved RBC storage solutions in use today. With the advent of component separation technologies RBC storage solutions evolved to optimize the maintenance of RBC viability during storage. A number of variants on the original citrate- and dextrose-based solutions have been developed, most notably the addition of adenine.[1] Nevertheless, in general, current storage solutions are designed to prevent plasma coagulation by using citrate; provide dextrose to support ongoing metabolism; and provide other nutrients, essential electrolytes, and nucleobases.

While current storage solutions provide critical components needed for RBC survival, cells still experience a

ABBREVIATIONS: DEHP = di(2-ethylhexyl)phthalate; HEL = hen egg lysozyme; MCP = monocyte chemoattractant protein; OVA = ovalbumin.

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Received for publication June 13, 2012; revision received November 12, 2012, and accepted November 16, 2012.

doi: 10.1111/trf.12106

TRANSFUSION 2013;53:2248-2257.

variety of alterations during storage, collectively referred to as the "storage lesion." Alterations in adenosine triphosphate (ATP), 2,3-diphosphoglycerate acid (2,3-DPG), and other metabolites have been observed during storage and may influence the RBC's ability to survive after transfusion and efficiently deliver O_2.[2-5] Additionally, RBCs lose the ability to handle ongoing oxidative stress and generation of reactive oxygen species during storage, which ultimately lead to oxidation of RBC lipids and proteins.[4,6-8] Unlike many other metabolic changes, protein and lipid oxidation are irreversible changes within an RBC. Such changes may not only compromise RBC function,[7,9] but may also directly stimulate the production of inflammatory mediators, with the potential to enhance recipient inflammation and alloimmunization after storage.[10-12]

Modern storage additive solutions (ASs) generally do not contain ingredients specifically targeted at the inhibition and/or detoxification of reactive oxidative species. To test the hypothesis that the addition of a known antioxidant to the RBC storage milieu would improve their storage, we studied the effects of ascorbic acid (vitamin C), one of the best-studied naturally occurring antioxidants.[13,14] To examine the potential impact of ascorbic acid on RBC storage, we utilized the HOD.FVB murine model of RBC storage.[11,15,16] This model utilizes donor mice that express a transgenic antigen specifically on RBCs. The antigen is a triple fusion protein with hen egg lysozyme (HEL) fused to ovalbumin (OVA) fused to Duffy (Fy^b variant)—HEL-OVA-Duffy (HOD). The presence of HOD on donor (but not recipient) RBCs allows detailed analysis of the recipient's humoral response to the HOD antigen. As HOD is present on RBCs from the donor strain, but is completely absent on recipient RBCs, it constitutes an alloantigenic challenge most similar to the transfusion of D+ RBCs to D– recipients. As with RhD, the rate of alloimmunization to transfused HOD RBCs is higher than that seen with blood group antigens that differ between donor and recipient by only a single amino acid. In addition to permitting analysis of alloimmunization, HOD contains the Fy3 epitope in the Duffy system, for which there is a monoclonal anti-Fy3 (MIMA-29) available. Staining peripheral blood with anti-Fy3 allows tracking and recovery studies without utilizing labeling procedures that may alter RBC biology during storage and after transfusion. Using the HOD.FVB model, we have recently reported that compared to fresh RBCs, transfusion of stored HOD.FVB RBCs results in reduced RBC viability and posttransfusion recovery, the induction of recipient cytokines, and increased humoral alloimmunization.[11,17,18] Herein, we report that the addition of ascorbic acid to a murine model of RBC storage had substantial benefits on stored RBCs regarding posttransfusion recovery and extent of humoral alloimmunization, but not induction of recipient cytokines.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, MD); FVB.HOD and FVB mice were bred by the Emory University Department of Animal Resources (Atlanta, GA). We have previously described FVB.HOD mice, which have RBC-specific expression of a model transgene consisting of HEL fused to the C-terminal half of OVA fused to the human Duffy antigen (Fy^b variant; HOD).[15] Recipient mice were 8- to 12-week-old females and all protocols were approved by the Emory University Institutional Animal Care and Use Committee.

Murine blood collection, storage conditions, and transfusion

Blood collection and transfusion were performed as previously described.[16] Briefly, HOD blood was collected into CPDA-1 by retroorbital bleeding. The CPDA-1 was obtained directly from FDA-approved human blood storage bags, and a final CPDA-1 concentration of 14% was used. The blood was centrifuged for 10 minutes at $324 \times g$, adjusted to a hematocrit (Hct) of 75% by removing supernatant, and stored in 500-μL Eppendorf tubes at 4°C for 14 days. RBCs were stored under three different storage conditions: 1) RBCs stored in CPDA-1 only without ascorbic acid and saline (standard storage), 2) RBCs stored in CPDA-1 and saline (saline only), and 3) RBCs stored in CPDA-1 with both saline and ascorbic acid (vitamin C). All recipient mice were C57BL/6 and received 75 μL of RBCs in a total volume of 500 μL of phosphate-buffered saline via the lateral tail vein (for cytokine experiments, 200 μL of RBCs was transfused). At the indicated time points, see below, peripheral blood (3 μL) was obtained by retroorbital bleed for determination of posttransfusion RBC recovery by flow cytometry.

Ascorbic acid stock preparation and addition

Ascorbic acid was purchased (Sigma-Aldrich, St Louis, MO; Product Number A5960), which is crystalline l-ascorbic acid at a purity of greater than or equal to 99.0%. A stock solution of 0.9% (wt/vol) NaCl was made and its pH was adjusted to 7.1 with small quantities of sodium hydroxide. Stock solutions of ascorbic acid were made in saline solution to final ascorbic acid concentrations of 2.06 mg/mL (11.71 mmol/L), 4.13 mg/mL (23.42 mmol/L), 6.19 mg/mL (35.13 mmol/L), and 41.25 mg/mL (234.22 mmol/L). All ascorbic acid–containing solutions were titrated to a pH of 7.1 with small quantities of sodium hydroxide after addition of ascorbic acid. The four stock solutions (and the saline control) were separately filtered through a 0.22-μm vacuum filter. The solutions were then added to stored mouse RBCs in a 1:9

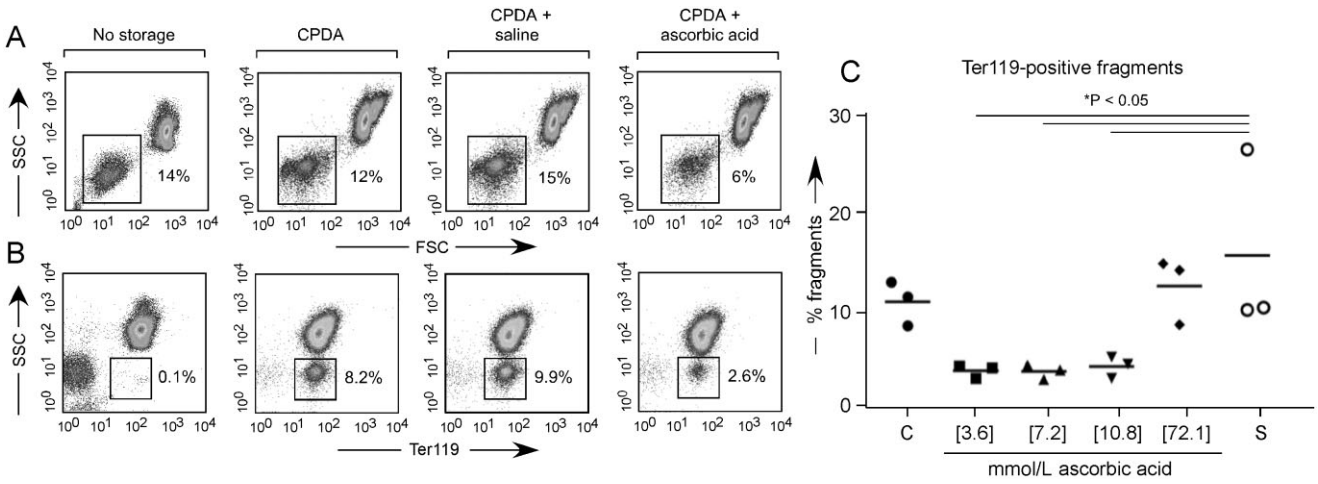

Fig. 1. Inclusion of ascorbic acid during storage inhibits generation of RBC microparticles. (A) FVB.HOD RBCs were incubated in CPDA-1, CPDA-1 plus saline, or CPDA-1 plus 3.6 mmol/L ascorbic acid in a saline solution for 14 days followed by flow cytometric analysis. Fragments are gated as indicated. (B) FVB.HOD RBCs were incubated with CPDA-1, CPDA-1 plus saline control, or CPDA-1 plus 3.6 mmol/L ascorbic acid in a saline solution for 14 days followed by staining with Ter119 and gating of Ter119-positive fragments as indicated. (C) Quantification of Ter119-positive fragments after storage of FVB.HOD RBCs with CPDA-1, CPDA-1 plus saline control, or CPDA-1 plus inclusion of ascorbic acid in a saline solution at the indicated concentrations for 14 days. C = storage in CPDA-1; S = storage in CPDA-1 plus saline. A and B show representative flow cytometry plots and the combined results of three separate experiments are shown in panel C. SSC = side scatter.

(vol/vol) ratio. Assuming a Hct of 75% before the addition of the AS, the final concentrations of ascorbic acid in the storage solutions were 0.63 mg/mL (3.6 mmol/L), 1.27 mg/mL (7.2 mmol/L), 1.90 mg/mL (10.8 mmol/L), and 12.69 mg/mL (72.1 mmol/L) corresponding to 61.5×, 123.1×, 184.6×, and 1230.8×, respectively, the normal human plasma ascorbic acid concentration of 10.3 µg/mL (58.55 µmol/L). Normal murine plasma ascorbic acid levels are approximately 90 µmol/L.[19]

Flow cytometry

RBC recovery was monitored by flow cytometry on samples acquired at 10 minutes, 30 minutes, 2 hours, and 24 hours after transfusion. Posttransfusion survival was calculated as a percentage of circulating RBCs after gating on donor RBCs by anti-Fy3 staining. Pretransfusion samples were also analyzed by flow cytometry and served as a baseline. The presence of HEL and Duffy epitopes was measured by staining with polyclonal anti-HEL antisera or monoclonal anti-Fy3 (MIMA 29 antibody, a gift from M. Reid and G. Halverson, New York Blood Center, New York, NY), under conditions previously described.[15] Goat anti-mouse immunoglobulins conjugated to allophycocyanin (Becton-Dickinson, San Jose, CA) was used as the secondary antibody. For crossmatching, sera were used at a 1:5 dilution; HOD.FVB RBCs and control FVB RBCs were used at a concentration of 3%. Pretransfusion RBC fragmentation was determined by staining stored RBCs with Ter119 conjugated to allophycocyanin (Becton-Dickinson) and examining Ter119-positive fragments by flow cytometry.

Statistical analysis

Two-way analysis of variance (ANOVA) with a Bonferroni posttest was performed utilizing computer software (Prism, GraphPad Software, San Diego, CA). A significant result was defined as a p value of 0.05 or less. In Figure 2, the differences between treatments at 24 hours were evaluated using the linear mixed effects (lme) function of the nonlinear mixed effects package of statistical software (R statistical language, http://www.r-project.org/) to perform a randomized complete blocks ANOVA.

RESULTS

Addition of ascorbic acid decreases formation of RBC microparticles during storage

RBCs were stored between 1 and 6°C for 14 days in CPDA-1 alone ("standard storage") or in CPDA-1 that was fortified with ascorbic acid solution starting at a concentration of 3.6 mmol/L ascorbic acid. Additional control RBCs were stored with CPDA-1 plus saline but without ascorbic acid ("saline control"). To analyze cellular decomposition, RBC units were analyzed by flow cytometry using forward and side scatter. This approach allows the visualization of intact RBCs in the upper right quadrant, whereas smaller material is visualized in the

lower left quadrant (Fig. 1). Storage of RBCs in ascorbic acid resulted in a 50% reduction in the quantity of smaller species (Fig. 1A). To assess the extent to which the smaller particles represented RBC microparticles, the specimens were stained with anti-TER119, which is an RBC-specific antigen on murine RBCs. Analyzing TER119 by side scatter allows a distinction between an intact RBC population (with higher side scatter) and RBC microparticles (with lower side scatter). The RBCs stored in ascorbic acid demonstrated a decrease in the accumulation of RBC microparticles by 69% compared to standard storage (CPDA-1 alone; Fig. 1B). The decrease in RBC microparticles was not an artifact of the saline in which the ascorbic acid was dissolved, as addition of the saline only RBCs did not decrease the accumulation of small material (Fig. 1A) or RBC microparticles (Fig. 1B). To test the dosage-dependent effects of ascorbic acid, a dose response was carried out from 3.6 to 72.1 mmol/L (each adjusted accordingly to neutral pH). Ter119+ RBC microparticles were quantified as above and were compared for different ascorbic acid concentrations. A similar effect of decreasing microparticle formation was seen from 3.6 to 10.8 mmol/L ascorbic acid RBCs compared to the standard storage or saline only RBCs. In contrast, the beneficial effects of ascorbic acid were lost at its highest concentration (72.1 mmol/L; Fig. 1C).

Addition of ascorbic acid to RBC storage increases posttransfusion recovery of RBCs

To test the effects of ascorbic acid on the posttransfusion recovery of RBCs, stored HOD antigen–positive RBCs were transfused into wild-type C57BL/6 recipients that do not express the HOD antigen. Recipients were bled at the indicated time points and the transfused RBCs were enumerated flow cytometrically by staining with anti-Fy3, which recognizes the donor RBCs that express the HOD antigen. This experiment was repeated three times, and each reproduction is shown (Fig. 2). In three of three experiments, the ascorbic acid RBCs demonstrated a significant increase in the RBCs' posttransfusion recovery at 24 hours compared to standard storage ($p = 0.01$), which was not an artifact of the saline, as no significant difference was present between standard storage and the saline-only control ($p = 0.28$). However, there was substantial variation in the early clearance kinetics in each of the three experiments, with the ascorbic acid or saline-only RBCs demonstrating variable recovery in the very early posttransfusion period (Fig. 2). Twenty-four hours after transfusion is the standard time to measure RBC recovery in humans, as rapid clearance occurs within this time period, and longer-stored human RBCs that survive for 24 hours have a life span equivalent to fresher-stored RBCs thereafter.[20] A similar trend is seen in these mice experiments, since progressive clearance is observed from 10

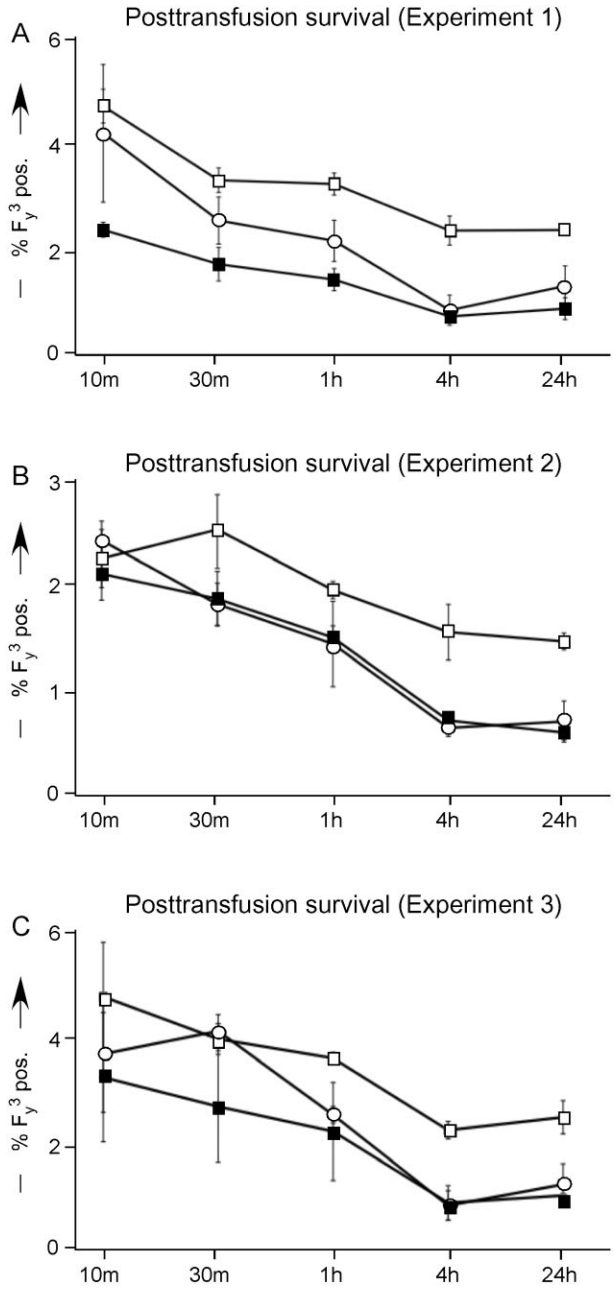

Fig. 2. Inclusion of ascorbic acid during storage enhances 24-hour posttransfusion recovery. (A-C) HOD.FVB RBCs were incubated with CPDA-1 (standard; –■–), CPDA-1 plus saline (saline control; –○–), or CPDA-1 plus 3.6 mmol/L ascorbic acid (–□–) for 14 days followed by transfusion and monitoring of RBC recovery at the indicated times points after transfusion. Gated cells represent the percentage of Fy3-positive cells recovered at each time point. A through C constitute three repetitions of the same experimental design. There are three animals in each group and error bars represent standard deviation. While there are inconsistent trends during the rapid clearance of early phase, trends normalize by 4 hours after transfusion and 24-hour recoveries are reproducible from experiment to experiment.

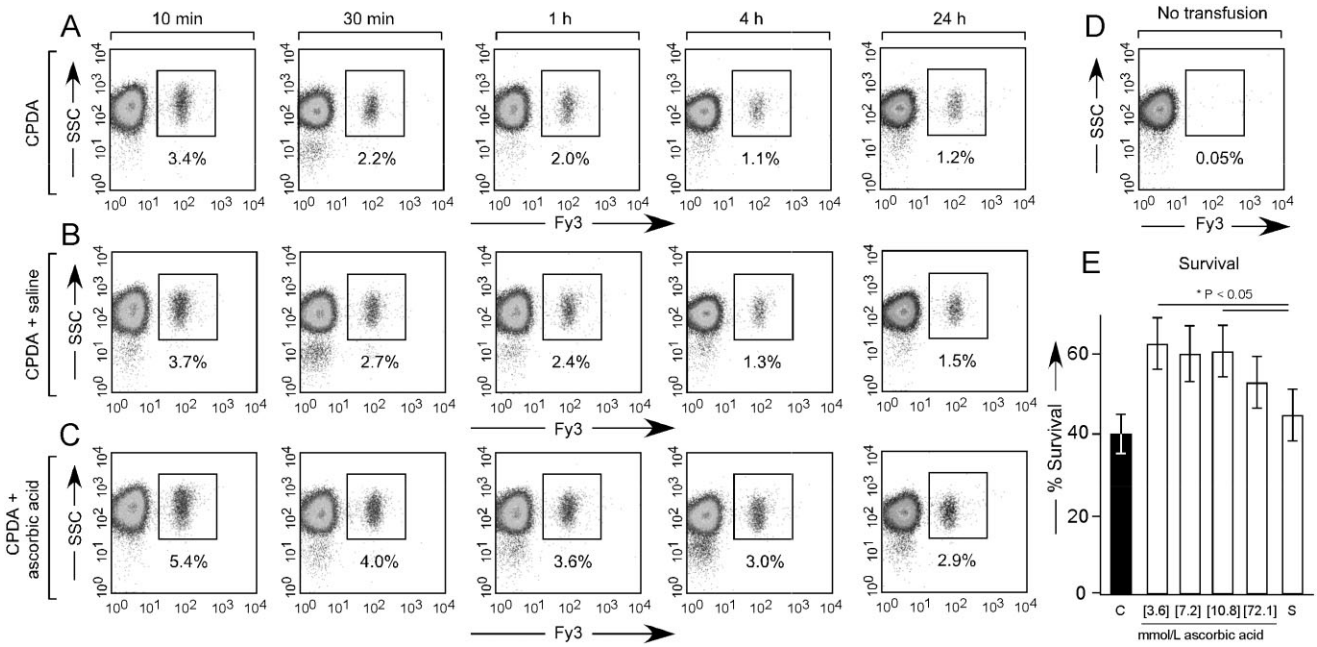

Fig. 3. Dose dependency of ascorbic acid enhancement of posttransfusion RBC survival. (A-C) Representative flow cytometry plots are shown for monitoring posttransfusion recovery with CPDA-1, CPDA-1 plus saline, or CPDA-1 plus 3.6 mmol/L ascorbic acid saline solution as indicated. (D) Negative control for gate determination (staining with anti-Fy3 on RBCs from untransfused wild-type recipients). (E) Quantification of the 24-hour posttransfusion survival of FVB.HOD RBCs after transfusion of stored cells in CPDA-1, CPDA-1 plus saline, or CPDA-1 plus the indicated concentrations of ascorbic acid (mmol/L) in saline for 14 days. C = storage in CPDA-1; S = storage in CPDA-1 plus saline; SSC = side scatter. Concentrations of ascorbic acid are in mmol/L. To allow combination of data, the change in percent recovery at 24 hours in each case was normalized to the 10-minute levels within the same experiment, thus providing an internal standard. Thus, percent survival represents normalized internal percent survival, not an overall percent recovery.

minutes to 4 hours after transfusion, but appears to stabilize between 4 and 24 hours.

Representative individual flow cytometry plots (which are combined in the recovery curves in Fig. 2) are shown in Figure 3. The gating strategy used to enumerate transfused HOD RBCs by Fy3 staining was validated by establishing that the gate is essentially empty in untransfused control mice (Fig. 3D). Similar to the dose effect of the ascorbic acid concentration on RBC fragmentation before transfusion, increasing concentrations of ascorbic acid resulted in increased RBC recovery (up to the 10.8 mmol/L concentration), whereas the beneficial effect of ascorbic acid on recovery was lost at 72.1 mmol/L (Fig. 3E). Together, these results demonstrate that the addition of an ascorbic acid solution can significantly improve the posttransfusion recovery of stored RBCs after transfusion.

Ascorbic acid does not decrease cytokine induction caused by transfusion of stored RBCs

We have previously reported that the transfusion of stored murine RBCs induces innate immune activation in the recipient in the form of a burst of cytokine secretion. As this effect was most strongly observed after the transfusion of 2 units of RBCs (200 µL of RBCs), additional animals in the current study received 2 units of either fresh or 14-day-old RBCs followed by serum collection 2 hours later. To investigate cytokine secretion, we focused on the two cytokines that gave the most significant response in previous studies, monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-6.[18] Similar to our previous reports, significant cytokine induction was seen with increased MCP-1 in mice receiving standard stored RBCs (CPDA-1 alone; S) compared to untransfused animals (NT; Fig. 4A), with a less pronounced but significant increase in IL-6 (Fig. 4B). No significant decrease in cytokine secretion was observed after the transfusion of the ascorbic acid RBCs, although there was a trend toward lower levels in the mice that were transfused with these RBCs. Moreover, there were two outliers among the mice that were transfused with the saline-only RBCs insofar as they demonstrated very high levels of IL-6 and MCP-1 (the same two mice had elevated levels of both cytokines). To avoid inducing baseline inflammation, pretransfusion bleeds were not obtained; thus, it is unclear if these outliers were inflamed due to underlying illness at baseline or if they had an unusual response to transfusion. While we

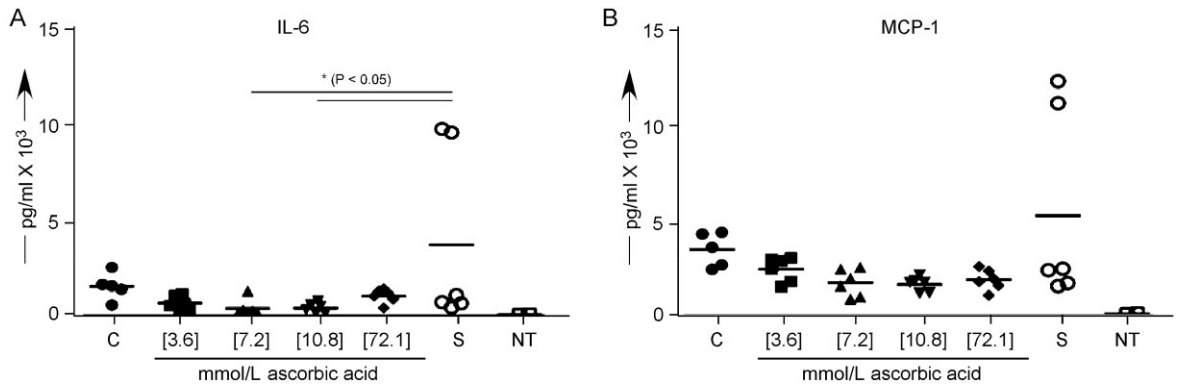

Fig. 4. Inclusion of ascorbic acid during storage does not significantly decrease transfusion-induced cytokine induction in recipients. Serum cytokines were measured 2 hours after transfusion of cells stored in CPDA-1, CPDA-1 plus saline, or CPDA-1 plus the indicated concentrations of ascorbic acid (mmol/L) in saline for 14 days for MCP-1 (A) or IL-6 (B). C = storage in CPDA-1; S = storage in CPDA-1 plus saline; NT = not transfused. Concentrations of ascorbic acid are in mmol/L. These data are the combined outcome of three separate experiments; horizontal bars represent the mean values.

cannot arbitrarily exclude these two outliers, they do not reflect what is seen with the other mice in the current study or in multiple previous animal studies.[18,21] Together, these data do not support a significant decrease in innate immune activation by the addition of ascorbic acid to the RBC storage solution.

Ascorbic acid mitigates the increase in alloimmunization observed with stored HOD.FVB RBCs

We have previously reported that transfusion of stored HOD.FVB RBCs results in increased recipient titers of anti-HOD; thus, the storage of HOD.FVB RBCs increases their immunogenicity upon transfusion. To test the effects of ascorbic acid on HOD antigen immunogenicity, serum was collected 2 weeks after transfusion and the titer of anti-HOD was measured by indirect immunofluorescence using HOD.FVB RBCs as targets. Wild-type FVB RBCs were used as negative controls to establish a baseline for the background signal. Mean fluorescent intensities (MFIs) of control RBCs were subtracted from MFI of HOD.FVB targets to establish an adjusted MFI. Substantial levels of antibody were seen in mice transfused with standard storage (CPDA-1 alone) and the control RBCs stored with CPDA-1 plus saline. As with previous studies, there was significant variability within groups (Fig. 5A). The addition of ascorbic acid from 3.6-10.8 mmol/L in the RBC storage solution significantly decreased the levels of alloimmunization, and the effect was lost in the RBCs that were stored in the presence of 72.1 mmol/L ascorbic acid (Fig. 5A). Representative flow cytometric plots are shown (Fig. 5B). These effects were not a nonspecific result of the saline in which the ascorbic acid was dissolved, as no effect was seen in saline-only controls.

DISCUSSION

While the ability to store RBCs for transfusion facilitates their use in a wide range of diseases, it is clear that stored RBCs undergo a series of biologic alterations that are collectively called "the storage lesion." These changes are important in determining the RBC's 24-hour posttransfusion recovery, although a causal connection between transfusing older RBCs and any adverse clinical effects, if any, has yet to be conclusively demonstrated.[22,23] Although stored at 4°C, RBCs are metabolically active living cells and during storage the oxidation of structural proteins, denaturation of hemoglobin, oxidation of lipids, and release of RBC microparticles all occur.[24-27] It has been hypothesized that oxidative damage is in part responsible for the functional consequences of the storage lesion. Based on these changes during storage, we hypothesized that the addition of a chemical antioxidant (i.e., ascorbic acid) would mitigate the storage lesion. We tested this hypothesis in a murine model of RBC storage and observed that the addition of supraphysiologic concentrations of ascorbic acid (3.6-10.8 mmol/L) in the storage solution resulted in improved 24-hour posttransfusion recovery, decreased formation of microparticles in the RBC unit before transfusion, and decreased immunogenicity of the transfused RBCs (with regard to a model alloantigen [HOD]). All of these beneficial effects were lost when the ascorbic acid concentration was increased to 72.1 mmol/L. None of these beneficial effects were observed when the RBCs were stored in saline alone without ascorbic acid.

The interpretation of the recipient secretion of cytokines after the transfusion of RBCs stored with or without ascorbic acid is somewhat complicated. Compared to standard storage (CPDA-1 alone), the levels of IL-6 and MCP-1 were slightly lower after the transfusion of ascorbic

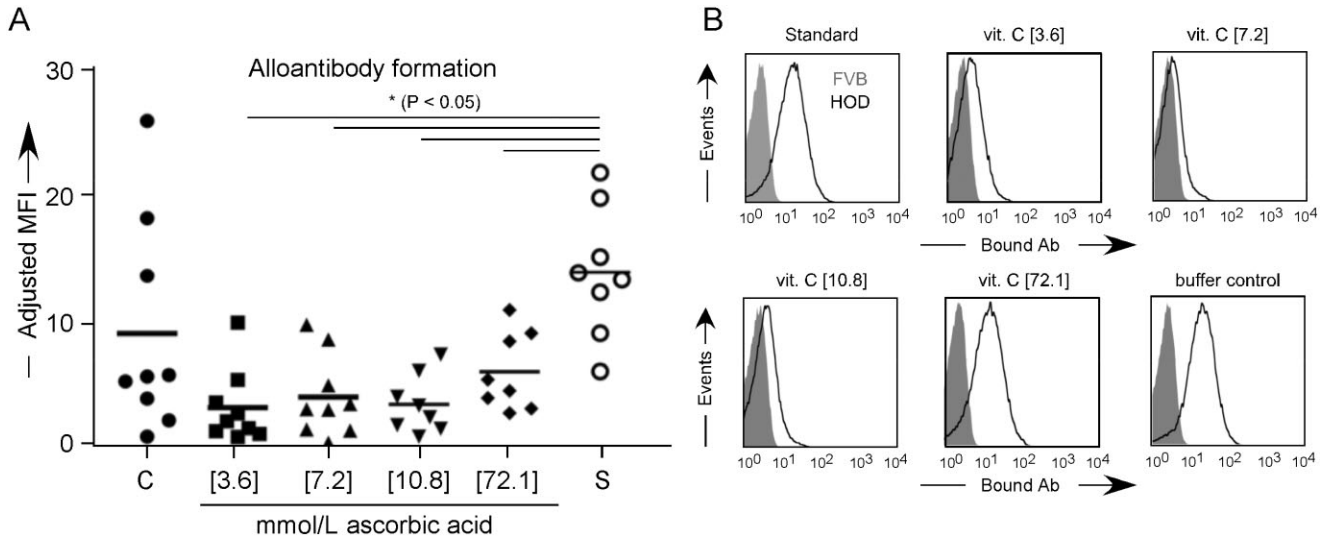

Fig. 5. Inclusion of ascorbic acid during storage reduces storage-induced increases in RBC alloimmunogenicity. (A) Quantification of the flow crossmatch MFI of serum isolated 14 days after transfusion of cells stored in CPDA-1, CPDA-1 plus saline, or CPDA-1 plus the indicated concentrations of vitamin C (vit. C; mmol/L) in saline. All conditions were stored for 14 days before transfusion. C = storage in CPDA-1; S = storage in CPDA-1 plus saline. These data are the combined results from three separate experiments and horizontal bars represent mean values. (B) Representative flow crossmatch with FVB (gray) or FVB.HOD (black) RBC targets with serum isolated 14 days after transfusion. Concentrations of ascorbic acid are in mmol/L. Ab = antibody.

acid–stored RBCs; however, cytokine levels were also decreased relative to standard storage by the addition of saline alone (without ascorbic acid). Thus, it seems unlikely that the ascorbic acid was responsible for the lower level of cytokine release by the recipient after RBC transfusion. Consistent with this interpretation, unlike each of the above variables (microparticles, recovery, and alloimmunization), the effect was still observed at the 72.1 mmol/L concentration of ascorbic acid. Taken together, we reject the interpretation that the addition of ascorbic acid has any benefit on the recipient's posttransfusion induction of cytokines.

The mechanism by which ascorbic acid neutralizes the increase in alloimmunogenicity conferred to HOD by RBC storage is unclear. It is worth noting that antisera response of B6 mice to the HOD antigen is directed almost exclusively to the HEL portion of HOD and not the Duffy antigens that are present.[28] We have also previously reported that HOD antigen does not decline overtime with storage under standard conditions, neither HEL nor Fy3 epitopes are decreased after 14 days storage.[11] We have argued that regulation of innate immunity at the time of transfusion regulates adaptive immune responses; however, of the innate immunity measured in this article, induction of cytokines does not seem to be significantly altered.

The issue of whether the beneficial effects that we have observed are due to ascorbic acid itself is also an important consideration. The saline-alone control was included to isolate ascorbic acid as a variable. However, the addition of ascorbic acid to the saline, and the subsequent required adjustment in pH, alters the osmolarity of the final solution to some extent. Moreover, there is alteration in the concentration of specific counterions (e.g., sodium). Accordingly, while the addition of ascorbic acid is the likely cause of the observed changes, we cannot unequivocally rule out the possibility that coincident factors are responsible. Nevertheless, the effects are not just an artifact of changes in volume or general components of the ascorbic acid carrier, as the RBCs stored in saline did not demonstrate the same changes during storage as did those stored in ascorbic acid. In fact, the RBCs stored in saline alone demonstrated similar findings to standard storage RBCs in most of the variables measured (with the notable exception of induction of cytokines in the recipient). It is also worth noting that these units of RBCs had both white blood cells (WBCs) and platelets (PLTs) present, accordingly, ascorbic acid could be having direct effects on cell types other than RBCs that then affect the RBCs.

The notion of adding ascorbic acid to stored RBCs is by no means a novel idea; indeed, the notion has a somewhat sordid history. As early as 1973, it was reported that the addition of ascorbic acid to stored RBCs resulted in a maintenance of 2,3-DPG.[29,30] It was subsequently discovered that it was not the ascorbic acid itself, but rather, oxalate contaminating the ascorbic acid that was responsible for the effect.[31,32] It is worth noting that 2,3-DPG is not known to correlate with RBC recoveries after transfusion, and while ATP does have a weak correlation to RBC

recoveries, ATP levels were not affected by oxalate.[33] Thus, it is unclear if this effect would translate to posttransfusion RBC recoveries; to the best of our knowledge, in vivo RBC recoveries were not reported in this context. Nevertheless, to investigate the possibility of oxalate contamination in this report, we developed a mass spectrometry assay for oxalate, which was undetectable in highly purified ascorbic acid being used in this study (data not shown). However, the limit of detection of the assay was 22 μmol/L, and 50 μmol/L concentrations of oxalate were shown to still prolong 2,3-DPG in stored RBCs. It is possible that oxalate levels are much lower (or absent) in the ascorbic acid utilized; however, given the limits of detection, we cannot unequivocally rule out that the effects seen herein are due to oxalate and not ascorbic acid.

It is unclear how ascorbic acid is exerting its effect(s) on stored RBCs. It is possible that ascorbic acid is acting on the outside of the RBC by interacting with reactive oxygen species generated in the liquid component of the RBCs by residual WBCs and/or PLTs. Ascorbic acid might also be acting in close proximity to or directly upon the RBC membrane. Ascorbic acid does not passively enter RBCs; thus, to affect the internal environment of the RBC, the ascorbic acid would have to be actively transported across the membrane. Two high-affinity ascorbic acid receptors have been described, SVCT1 and SVCT2.[34] While SVCT2 is present in erythroblasts, it is largely absent from mature RBCs.[35] However, mature RBCs still take up low levels of radiolabeled ascorbate, which occurs when dehydroascorbic acid is imported into the RBC by glucose transporters (GLUT) followed by conversion to ascorbic acid inside the RBC by glutathione.[36] Mature human RBCs uptake dehydroascorbic acid (through GLUT1) at a higher level than do murine RBCs (which use GLUT4);[36] however, murine RBCs do have low baseline absorption of dehydroascorbic acid. This represents a difference between human and mouse RBCs and may be related to the fact that for the former, ascorbic acid represents an essential vitamin. In contrast, mice synthesize ascorbic acid naturally. Although we have attempted to model human storage in our mouse system, there are additional differences that also must be taken into consideration. While RBCs are processed in a fashion that mimics human RBC processing, Eppendorf tubes are used for storage, which differs from human blood bags. CPDA-1 from human blood bags was used in collection so as to capture di(2-ethylhexyl)phthalate (DEHP) that is present in human bags; however, additional DEHP that leaches from the bag is absent. As DEHP is known to affect RBCs, it is important to note that this effect may be diminished or absent in the current studies.[37] Moreover, mice clearly differ from humans in multiple ways. Ultimately, human studies will be required to determine if human RBCs have different responses to ascorbic acid addition during storage than do mouse RBCs.

Ascorbic acid represents one of the most well-studied naturally occurring antioxidants.[13,14] Indeed, previous studies have demonstrated that ascorbic acid can significantly reduce cellular oxidation,[14,38,39] thereby enhancing cellular viability and improving cellular function (of non-RBC populations). The ability of ascorbic acid solution to enhance RBC recovery, decrease microparticle formation, and reduce alloimmunization demonstrates that RBCs can also benefit from exposure to ascorbic acid. These data also suggest that RBC oxidation during storage contributes to the reduced recovery and enhanced alloimmunogenicity of blood stored without ascorbic acid. The loss of benefit at higher doses of ascorbic acid is also consistent with this hypothesis as ascorbic acid is well known to have paradoxical prooxidant activity at higher doses.[40] Consistent with this notion, it has been reported that RBCs can be stored for longer periods of time under anaerobic conditions[41-43] in which oxygen is absent and oxidation is presumably decreased. However, it is worth noting that it is also possible that these effects are caused by other alterations besides the depletion of oxygen.[44]

Although storage solutions provide key nutrients required for RBC recovery, they do not possess factors known to actively inhibit oxidation. The results of this study demonstrate that inclusion of ascorbic acid, a well-known naturally occurring antioxidant,[13,14] during RBC storage significantly enhances posttransfusion recovery and reduces alloimmunization in murine recipients after transfusion. Future studies should not only focus on the biochemical changes that may occur during RBC storage with ascorbic acid, but should also examine other known antioxidants, to evaluate the general potential of antioxidant inclusion during storage on mitigating the undesirable effects of storage on RBCs. The murine system represents a tractable platform in which to rapidly generate and test hypotheses; as always, subsequent focused studies in humans will be required to test these hypotheses in human RBC storage systems.

ACKNOWLEDGMENT

We thank Doug Bolgiano for his help with statistical analysis and review of the manuscript.

CONFLICT OF INTEREST

JCZ, AFP, and MHY have disclosed technology contained herein to their respective intellectual property offices. The other authors have no conflicts of interest to declare.

REFERENCES

1. Hess JR. An update on solutions for red cell storage. Vox Sang 2006;91:13-9.
2. Gary-Bobo CM, Solomon AK. Properties of hemoglobin solutions in red cells. J Gen Physiol 1968;52:825-53.

3. Gelderman MP, Yazer MH, Jia Y, Wood F, Alayash AI, Vostal JG. Serial oxygen equilibrium and kinetic measurements during RBC storage. Transfus Med 2010;20:341-5.
4. Hogman CF, Meryman HT. Storage parameters affecting red blood cell survival and function after transfusion. Transfus Med Rev 1999;13:275-96.
5. Raat NJ, Ince C. Oxygenating the microcirculation: the perspective from blood transfusion and blood storage. Vox Sang 2007;93:12-8.
6. Chaudhary R, Katharia R. Oxidative injury as contributory factor for red cells storage lesion during twenty eight days of storage. Blood Transfus 2012;10:59-62.
7. Dumaswala UJ, Zhuo L, Jacobsen DW, Jain SK, Sukalski KA. Protein and lipid oxidation of banked human erythrocytes: role of glutathione. Free Radic Biol Med 1999;27: 1041-9.
8. Trotta RJ, Sullivan SG, Stern A. Lipid peroxidation and haemoglobin degradation in red blood cells exposed to t-butyl hydroperoxide. The relative roles of haem- and glutathione-dependent decomposition of t-butyl hydroperoxide and membrane lipid hydroperoxides in lipid peroxidation and haemolysis. Biochem J 1983;212:759-72.
9. Knight JA, Voorhees RP, Martin L, Anstall H. Lipid peroxidation in stored red cells. Transfusion 1992;32:354-7.
10. Lysenko L, Mierzchala M, Gamian A, Durek G, Kubler A, Kozlowski R, Sliwinski M. The effect of packed red blood cell storage on arachidonic acid and advanced glycation end-product formation. Arch Immunol Ther Exp (Warsz) 2006;54:357-62.
11. Hendrickson JE, Hod EA, Spitalnik SL, Hillyer CD, Zimring JC. Storage of murine red blood cells enhances alloantibody responses to an erythroid-specific model antigen. Transfusion 2010;50:642-8.
12. Mangalmurti NS, Chatterjee S, Cheng G, Andersen E, Mohammed A, Siegel DL, Schmidt AM, Albelda SM, Lee JS. Advanced glycation end products on stored red blood cells increase endothelial reactive oxygen species generation through interaction with receptor for advanced glycation end products. Transfusion 2010;50:2353-61.
13. Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, Chen S, Corpe C, Dutta A, Dutta SK, Levine M. Vitamin C as an antioxidant: evaluation of its role in disease prevention. J Am Coll Nutr 2003;22:18-35.
14. Khassaf M, McArdle A, Esanu C, Vasilaki A, McArdle F, Griffiths RD, Brodie DA, Jackson MJ. Effect of vitamin C supplements on antioxidant defence and stress proteins in human lymphocytes and skeletal muscle. J Physiol 2003; 549:645-52.
15. Desmarets M, Cadwell CM, Peterson KR, Neades R, Zimring JC. Minor histocompatibility antigens on transfused leukoreduced units of red blood cells induce bone marrow transplant rejection in a mouse model. Blood 2009;114:2315-22.
16. Gilson CR, Kraus TS, Hod EA, Hendrickson JE, Spitalnik SL, Hillyer CD, Shaz BH, Zimring JC. A novel mouse model of red blood cell storage and posttransfusion in vivo survival. Transfusion 2009;49:1546-53.
17. Hendrickson JE, Hod EA, Cadwell CM, Eisenbarth SC, Spiegel DA, Tormey CA, Spitalnik SL, Zimring JC. Rapid clearance of transfused murine red blood cells is associated with recipient cytokine storm and enhanced alloimmunogenicity. Transfusion 2011;51: 2445-54.
18. Hod EA, Zhang N, Sokol SA, Wojczyk BS, Francis RO, Ansaldi D, Francis KP, Della-Latta P, Whittier S, Sheth S, Hendrickson JE, Zimring JC, Brittenham GM, Spitalnik SL. Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. Blood 2010;115:4284-92.
19. Kim H, Bae S, Yu Y, Kim Y, Kim HR, Hwang YI, Kang JS, Lee WJ. The analysis of vitamin C concentration in organs of gulo(-/-) mice upon vitamin C withdrawal. Immune Netw 2012;12:18-26.
20. Luten M, Roerdinkholder-Stoelwinder B, Schaap NP, de Grip WJ, Bos HJ, Bosman GJ. Survival of red blood cells after transfusion: a comparison between red cells concentrates of different storage periods. Transfusion 2008;48: 1478-85.
21. Hendrickson JE, Hod EA, Hudson KE, Spitalnik SL, Zimring JC. Transfusion of fresh murine red blood cells reverses adverse effects of older stored red blood cells. Transfusion 2011;51:2695-702.
22. Triulzi DJ, Yazer MH. Clinical studies of the effect of blood storage on patient outcomes. Transfus Apher Sci 2010;43: 95-106.
23. van de Watering L. Red cell storage and prognosis. Vox Sang 2011;100:36-45.
24. Karon BS, Van Buskirk CM, Jaben EA, Hoyer JD, Thomas DD. Temporal sequence of major biochemical events during blood bank storage of packed red blood cells. Blood Transfus 2012;10:1-9.
25. Kriebardis AG, Antonelou MH, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS. Membrane protein carbonylation in non-leukodepleted CPDA-preserved red blood cells. Blood Cells Mol Dis 2006;36: 279-82.
26. Kriebardis AG, Antonelou MH, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS. Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells. J Cell Mol Med 2007;11:148-55.
27. Kriebardis AG, Antonelou MH, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS. RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components. Transfusion 2008;48:1943-53.
28. Hudson KE, Lin E, Hendrickson JE, Lukacher AE, Zimring JC. Regulation of primary alloantibody response through antecedent exposure to a microbial T-cell epitope. Blood 2010;115:3989-96.

29. Wood L, Beutler E. The effect of ascorbate and dihydroxyacetone on the 2,3-diphosphoglycerate and ATP levels of stored human red cells. Transfusion 1974;14:272-7.
30. Wood LA, Beutler E. The effect of ascorbate on the maintenance of 2,3-diphosphoglycerate (2,3-DPG) in stored red cells. Br J Haematol 1973;25:611-18.
31. Beutler E. The "ascorbate" effect on 2,3-DPG is known to be due to oxalate [letter]. Vox Sang 2004;86:199; author reply 200.
32. Kandler R, Grode G, Symbol R, Hickey G. Oxalate is the active component that produces increased 2,3-DPG in ascorbate stored red cells [letter]. Transfusion 1986;26:563.
33. Beutler E, Forman L, West C. Effect of oxalate and malonate on red cell metabolism. Blood 1987;70:1389-93.
34. Savini I, Rossi A, Pierro C, Avigliano L, Catani MV. SVCT1 and SVCT2: key proteins for vitamin C uptake. Amino Acids 2008;34:347-55.
35. May JM, Qu ZC, Qiao H, Koury MJ. Maturational loss of the vitamin C transporter in erythrocytes. Biochem Biophys Res Commun 2007;360:295-8.
36. Montel-Hagen A, Kinet S, Manel N, Mongellaz C, Prohaska R, Battini JL, Delaunay J, Sitbon M, Taylor N. Erythrocyte Glut1 triggers dehydroascorbic acid uptake in mammals unable to synthesize vitamin C. Cell 2008;132:1039-48.
37. Draper CJ, Greenwalt TJ, Dumaswala UJ. Biochemical and structural changes in RBCs stored with different plasticizers: the role of hexanol. Transfusion 2002;42:830-5.
38. Yang CC, Hsu SP, Wu MS, Hsu SM, Chien CT. Effects of vitamin C infusion and vitamin E-coated membrane on hemodialysis-induced oxidative stress. Kidney Int 2006;69:706-14.
39. Heitzer T, Just H, Munzel T. Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. Circulation 1996;94:6-9.
40. Arrigoni O, De Tullio MC. Ascorbic acid: much more than just an antioxidant. Biochim Biophys Acta 2002;1569:1-9.
41. Yoshida T, Shevkoplyas SS. Anaerobic storage of red blood cells. Blood Transfus 2010;8:220-36.
42. Dumont LJ, Yoshida T, AuBuchon JP. Anaerobic storage of red blood cells in a novel additive solution improves in vivo recovery. Transfusion 2009;49:458-64.
43. Yoshida T, AuBuchon JP, Tryzelaar L, Foster KY, Bitensky MW. Extended storage of red blood cells under anaerobic conditions. Vox Sang 2007;92:22-31.
44. Hess JR. Storage of red blood cells under anaerobic conditions [letter]. Vox Sang 2007;93:183.