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Red blood cells are damaged by intraoperative blood salvage *via* Ca^{2+}-dependent and -independent mechanisms

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ABSTRACT

Aims: Intraoperative blood salvage (IBS) is associated with shortened lifespan of red blood cells (RBCs). This study aims to examine how salvaged RBCs are compromised during IBS.

Main methods: Thirty patients who underwent vertebra surgery with IBS were included in the study. To examine possible mechanisms of IBS-induced injury, both fresh and salvaged RBCs from each patient were mixed with plasma, the Ca^{2+} ionophore ionomycin or mannitol-adenine-phosphate (MAP) solution (n = 10 patients per condition). Binding of Fluo-3 and/or Annexin V by RBCs was measured.

Key findings: The percentage of Fluo-3-binding RBCs in salvaged samples was 2.83 ± 0.76%, which increased to 15.34 ± 5.99% after 48-h incubation in plasma. These percentages were significantly higher than those observed with fresh RBCs ($P < 0.01$). Ionomycin dose-dependently increased the percentage of Fluo-3-binding RBCs in salvaged samples, while MAP solution decreased it. Incubating salvaged RBCs in plasma for 48 h increased the percentage of Fluo-3-positive spherocytes from 0.8 ± 0.6% to 11.35 ± 3.96%, and this increase was blocked by MAP solution. Ionomycin increased the percentage of RBCs binding both Fluo-3 and Annexin V, while MAP decreased this percentage. The percentage of Annexin V-binding RBCs was also higher in salvaged samples than in fresh samples, but this percentage was unaffected by either ionomycin or MAP solution.

Significance: Our results suggest that IBS induces a postponed RBC damage by inducing spherocyte formation, which likely reflects Ca^{2+} entry induced by energy exhaustion, as well as by exposing phosphatidylserine on the RBC surface, which likely occurs *via* Ca^{2+} entry and *via* Ca^{2+}-independent pathways.

1. Introduction

Intraoperative blood salvage (IBS) has been widely used in the clinic [1]. While it can significantly reduce the need for red blood cell (RBC) transfusions during 24 h [2], its ability to reduce the need for transfusions over longer periods is doubtful [3–11]. We found that hemoglobin levels in surgical patients over the first 3 postoperative days were significantly lower in those who underwent IBS than in those who did not, and that salvaged RBC volume was an independent risk factor for this hemoglobin decrease [12]. The available evidence, then, suggests that IBS damages RBCs and leads to their rapid elimination after redelivery to the patient, as a result, IBS currently is not guaranteed to reduce transfusion requirements over longer periods.

Damaged RBCs may undergo hemolysis or be engulfed by phagocytes, then cleared from the body. Our comparison of IBS and non-IBS patients showed that levels of hemoglobin in plasma did not increase after redelivery of RBCs [13], leading us to propose that IBS shortens the lifespan of salvaged RBCs not by inducing hemolysis but by triggering RBC engulfment by phagocytes. In the case of aging RBCs, phagocyte engulfment can occur by a receptor-dependent mechanism, which requires the presence of exposed phosphatidylserine (PS) on the RBC surface [14,15]; or by a receptor-independent mechanism, which requires irreversible morphological transformation into a rigid RBC [13] like spherocyte. Both mechanisms can involve intracellular Ca^{2+} overload and energy depletion [14–17].

Our studies with IBS show that salvaged RBCs are similar to aging

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RBCs in that they can feature energy depletion [16] and expose PS on their surface [18]. These findings led us to hypothesize that salvaged RBCs may ultimately be engulfed by phagocytes *via* the same mechanisms as aging RBCs. Using Fluo-3, a fluorescence indicator that binds to intracellular Ca^{2+} [17], we examined this hypothesis by looking at mechanisms dependent and independent of Ca^{2+} and energy depletion. The insights from this work may help develop strategies to prolong the lifespan of salvaged RBCs.

2. Material and methods

2.1. Patients

This prospective study involved a consecutive series of 30 patients who underwent selective spinal surgery involving IBS between June 1, 2015 and November 30, 2015 at the Affiliated Hospital of Zunyi Medical University (Zunyi City, China). Patients were eligible if they were between 18 and 70 years old and had American Society of Anesthesiologists (ASA) Physical Status I-III. Patients were excluded if they had one of the following conditions: a serious disorder of the coronary, peripheral and/or carotid arteries; low hemoglobin (< 8 g/dL); hematologic or malignant disease; diabetes; untreated hypertension (diastolic blood pressure > 95 mmHg); renal or liver dysfunction; sickle cell anemia; or prophylactic anticoagulant therapy. Patients who had received RBC transfusion before study enrollment were also excluded.

This study was approved by the Ethics Committee of Zunyi Medical University, and participants gave written, informed consent prior to enrollment. This trial has been registered in the China Clinical Trial Registry (ChiCTR-OCH-14005140). All methods were performed in accordance with the corresponding references.

2.2. Interventions

RBCs were salvaged during surgery using an autologous blood recovery system (3000P, Jingjing Medical Equipment, Beijing, China) as described [10]. Briefly, blood shed from the wound was collected, anticoagulated with 25 U/mL heparin in 0.9% normal saline, and washed in 250-mL centrifuge bottles with 1.5–2 L of 0.9% normal saline. The relatively large wash volumes were required in order to wash the cells until the supernatant was clear, as per recommendations of the manufacturer of the blood recovery system.

RBCs were harvested from salvaged and fresh arterial blood samples from the same patient. (Fresh samples were taken before redelivery of salvaged RBCs.) Blood samples were centrifuged at 800*g* for 5 min. The supernatant was discarded and the cell pellet was resuspended in phosphate-buffered saline (PBS) to a concentration of 5×10^6 cells/mL.

2.3. RBC incubation in plasma

To mimic the entry of IBS red blood cells into the human body, salvaged cells from 10 patients were mixed with plasma (from the same patient) to give a final hematocrit of 35%, and cultured at 37 °C for 48 h on a platform rotating at 80 rpm [16]. Fresh RBCs from the same patient served as a control. Plasma and fresh RBCs were obtained from each patient before redelivery of salvaged RBCs. RBC morphology was examined under a phase contrast microscope (DP70, Olympus, Hauppauge, NY, USA) and a laser-scanning confocal microscope (Nikon, Melville, NY, USA). The percentage of spherocytes (cells without a biconcave discoid shape) was calculated from counts of at least 500 cells under the confocal microscope. Percentages of Annexin V- and/or Fluo-3-binding cells were determined using flow cytometry (see below).

2.4. RBC exposure to ionomycin

To examine whether IBS induces PS exposure on RBCs by increasing cytosolic Ca^{2+} concentration, salvaged and fresh RBCs were seeded separately into three, 6-cm centrifuge tubes, after which S-MEM (Gibco) containing 1.0 mmol/L $CaCl_2$ was added. Then the Ca^{2+} ionophore ionomycin [18] (Sigma-Aldrich, Saint Louis, MO, USA) was added to a final concentration of 0, 0.05, or 0.1 μM. The cells were plated and cultured at 37 °C on a platform rotating at 80 rpm. After 2 h of culture, cells were harvested and diluted to 1×10^6 cells/mL using Hank's Balanced Salt Solution to identify Annexin V- and/or Fluo-3-binding cells using flow cytometry (see below).

2.5. RBC exposure to mannitol-adenine-phosphate (MAP) solution

IBS may deplete energy stores in RBCs [16] and thereby activate Ca^{2+} entry [14,15]. If true, then boosting energy production should reduce Ca^{2+} entry and thus PS exposure, helping prolong salvaged RBC lifespan after redelivery to the patient. Salvaged RBCs and fresh plasma from the same patient were mixed to give a final hematocrit of 35% (as in the experiment described in "*RBC incubation in plasma*"), then half the mixture was added to an equal volume of MAP, and another half was added to an equal volume of 0.9% normal saline. The resulting mixtures were incubated for 24 h in a 37 °C water bath on a shaker. Both adenine nucleotide levels in RBCs and Annexin V- and/or Fluo-3-binding cells were analyzed before and after incubation for 24 h (see below).

2.6. Analysis of Annexin V- and/or Fluo-3-binding cells

Fluo-3AM can be used to bind intracellular Ca^{2+} [32]. Fluo-3AM (Invitrogen) was added to salvaged and fresh RBCs to a final concentration of 4.0 μg/mL, and the mixture was incubated at 37 °C for 30 min. The suspension was centrifuged at 800*g* for 5 min, the supernatant was discarded, and cells were first washed in cold PBS, then resuspended in 1× Binding Buffer (BD Bioscience). APC-conjugated Annexin V [10] (3.0 μL; BD Bioscience) was added to the resuspended cells (100.0 μL, 1×10^5 cells), and the mixture was incubated at room temperature for 15 min in the dark. To this mixture was added 400.0 μL 1× Binding Buffer, and then flow cytometry analysis of at least 50,000 events was performed using a Calibur flow cytometer (BD) equipped with Flowjo software (Tree Star, Ashland, OR, USA) (Fig. 1a, b).The instrument was calibrated daily using Rainbow Beads (BD).

Cells stained in this way were also examined for morphological changes. An aliquot of cells was dropped on a glass slide, overlaid with a coverslip and analyzed using laser-scanning confocal microscopy (Nikon; Fig. 1c).

2.7. Adenine nucleotide level and energy charge

The energy state of RBCs can be analyzed by assaying levels of adenosine tri-, di- and monophosphate (ATP, ADP and AMP) [16].RBC suspensions were diluted with an equal volume of distilled water, and mixed for 10 min with 0.5 mL perchloric acid (1.6 mol/L). The suspension was centrifuged for 10 min at 12,000*g* at 4 °C, the protein-free supernatant was neutralized using 1 mol/L K_2CO_3, and the mixture was centrifuged again to remove $KClO_4$ precipitates. Levels of ATP, ADP and AMP in the clarified supernatant were assayed using high-performance liquid chromatography (Agilent 1100, San Diego, CA, USA) and commercial adenine nucleotide assay kits (Sigma-Aldrich). Total adenine nucleotide concentration was derived from the sum of ATP, ADP, and AMP.

2.8. Statistical analysis

Data were analyzed using SAS 9.1 (SAS Institute, Cary, NC, USA) and reported as mean ± standard deviation. All data were used in all analyses; no data points were omitted. Differences between groups were assessed for significance using one-way analysis of variance. The paired

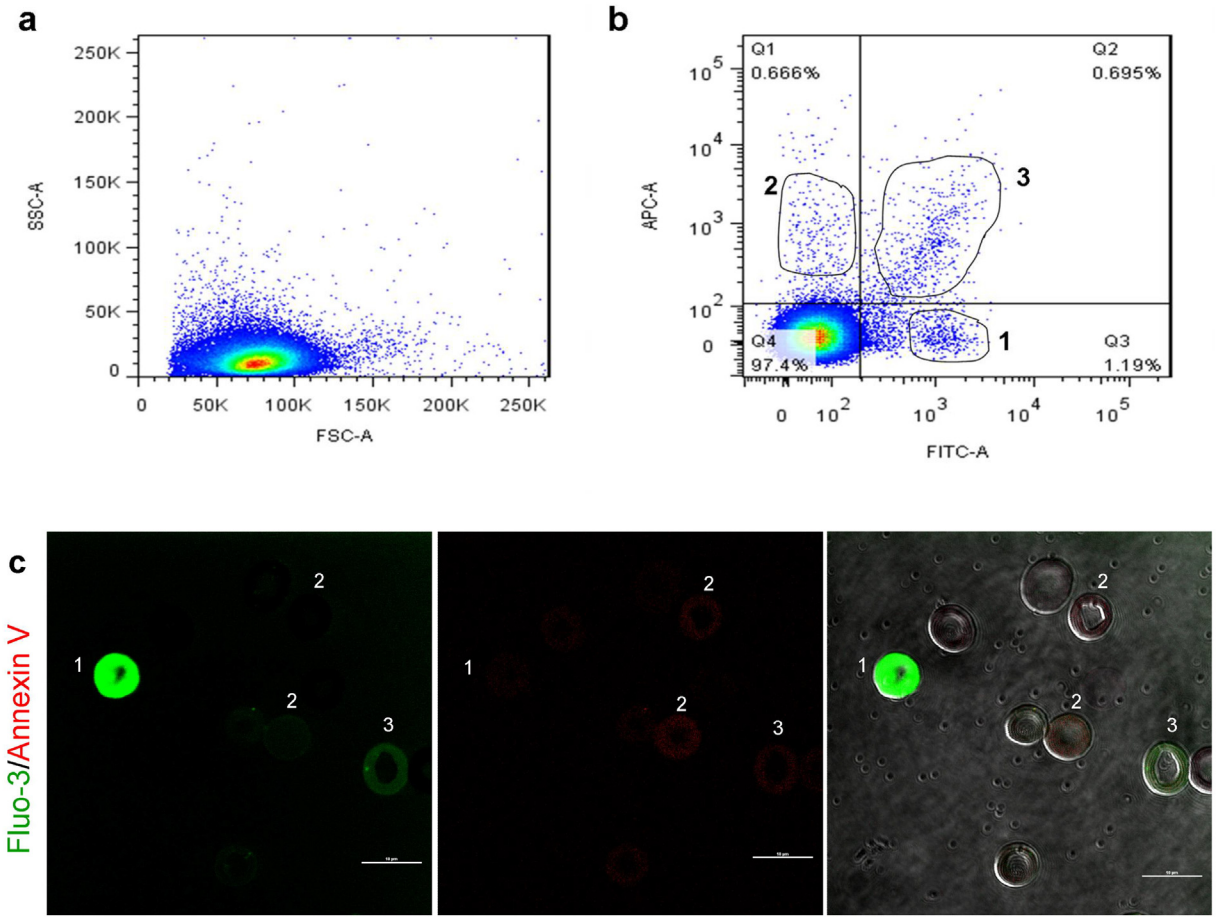

Fig. 1. Flow cytometry to assay intracellular Ca^{2+} levels and phosphatidylserine (PS) exposure. (a) Red blood cells (RBCs) were gated in dot plots of forward scatter (FSC) *vs* side scatter (SSC). (b) Representative results are shown for fresh blood. (c) Representative confocal fluorescence micrographs showing intracellular Ca^{2+} (green) and PS exposure (red). Cell 1 is an RBC with intracellular Ca^{2+}; cell 2, an RBC with exposed PS; and cell 3, an RBC with both intracellular Ca^{2+} and exposed PS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Student's *t*-test was used to assess the significance of differences between fresh and salvaged RBCs incubated in plasma, ionomycin, and MAP solution or 0.9% normal saline. $P < 0.05$ was considered to indicate statistical significance.

3. Results

The 30 patients analyzed in this study had the clinical and demographic characteristics shown in Table 1. The group was divided into three treatment subgroups of 10 patients each (see Material and methods).

3.1. RBC incubation in plasma: IBS damages RBCs

Fresh blood samples contained Fluo-3-positive RBCs (Zone 1 in Fig. 1b), Annexin V-positive RBCs (Zone 2 in Fig. 1b) and double-positive cells (Zone 3 in Fig. 1b). All of these cells in salvaged samples were identified by confocal microscopy (Fig. 1c: Fluo-3-positive, cell 1; Annexin V-positive, cell 2; double-positive, cell 3).

Morphology of salvaged RBCs remained normal after IBS, but it changed after 24 and 48 h of incubation (Fig. 2a, b). Based on flow cytometry, salvaged blood samples contained the same three populations but in significantly higher proportions than fresh samples before incubation (all $P < 0.01$): Fluo-3-positive binding cells, 2.83 ± 0.76% *vs* 1.57 ± 0.33%; Annexin V-positive, 1.52 ± 0.92% *vs* 0.76 ± 0.33%; and double-positive cells, 0.17 ± 0.11% *vs* 0.11 ± 0.04%. Larger differences were observed after 48-h incubation (all $P < 0.001$), when the three respective percentages in salvaged samples were 15.34 ± 5.99%, 3.40 ± 0.59% and 1.05 ± 0.26% (Fig. 2c–e). At this time point, the proportion of Fluo-3-binding cells in salvaged samples was 5-fold the proportion of Annexin V-binding cells and 15-fold the proportion of double-positive cells.

The proportion of spherocytes was significantly higher in salvaged samples than in fresh ones before incubation (0.8 ± 0.6% *vs* 0 ± 0%)

Table 1
Clinical and demographic characteristics of enrolled patients who underwent intraoperative blood salvage.

Characteristic	RBC treatment procedure[a]		
	Plasma (n = 10)	Ionomycin (n = 10)	MAP solution (n = 10)
Male/Female	6/4	5/5	6/4
Age, yr	42 ± 8	46 ± 10	44 ± 10
Weight, kg	58 ± 9	57 ± 7	59 ± 9
Height, cm	165 ± 5	170 ± 7	166 ± 6
Site of vertebra surgery, n			
Cervical	1	1	1
Thoracic	2	3	1
Lumbar	7	6	8
Surgery duration, h	3.45 ± 0.65	4.02 ± 1.24	3.72 ± 1.02
Salvaged red blood cells, mL	595 ± 138	714 ± 315	662 ± 280

[a] Patients were assigned to one of three treatment groups, in which matched samples of fresh and salvaged red blood cells (RBCs) were treated as described in Material and methods.

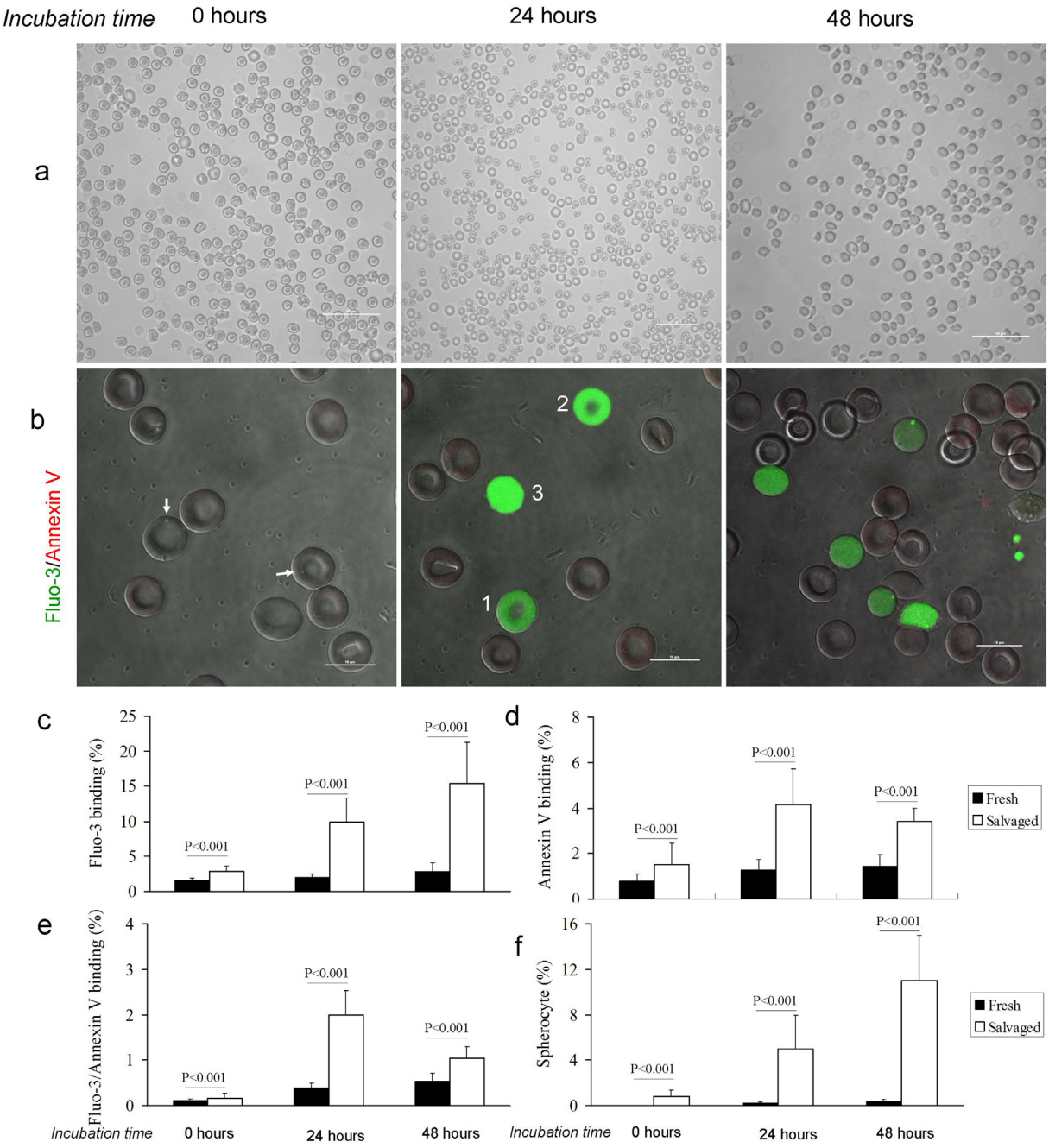

Fig. 2. Effects of incubating salvaged and fresh red blood cells from the same patient in plasma. (a) Morphology of salvaged red blood cells, based on phase contrast microscopy. (b) Salvaged cells were stained with Fluo-3 (green) and Annexin V (red). Intracellular Ca^{2+} entry (white arrow) was observed at baseline. Erythrocytes showing intracellular Ca^{2+} entry lost their biconcave discoid shape (cells 1–3) by 24 h of incubation and became spherocytes by 48 h. (c–f) Percentages of RBCs positive for Fluo-3, Annexin V, or both. Also shown are percentages of RBCs that were spherocytes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and after incubation for 24 h (5.12 + 3.01% *vs* 0.20 ± 0.12%) or 48 h (11.35 + 3.96% *vs* 0.41 ± 0.15%) (all P < 0.001, Fig. 2f). Fluorescence microscopy showed all spherocytes to be Fluo-3-positive (Fig. 2b).

In salvaged samples, proportions of Fluo-3-positive cells correlated with the proportion of spherocytes after incubation for 24 h (r = 0.84, P < 0.01) and 48 h (r = 0.92, P < 0.01).

3.2. RBC exposure to ionomycin: IBS damages RBCs via Ca^{2+}-dependent and -independent mechanisms

The proportion of Fluo-3-binding cells was significantly higher in salvaged RBCs than in fresh RBCs at all ionomycin concentrations tested (all P < 0.001), and the proportion increased with ionomycin dose (Fig. 3a): in the absence of ionomycin, 8.88 ± 2.86% *vs* 2.59 ± 0.77%; 0.05 μM ionomycin, 19.77 ± 9.24% *vs* 3.52 ± 1.39%; and 0.10 μM ionomycin, 42.72 ± 15.37% *vs* 4.25 ± 1.51%. Similar results were observed for the proportion of RBCs positive for both Fluo-3 and Annexin V (all P < 0.001, Fig. 3c): in the absence of ionomycin, 1.58 ± 1.20% *vs* 0.21 ± 0.11%; 0.05 μM ionomycin, 2.03 ± 1.82 *vs* 0.28 ± 0.12%; and 0.10 μM ionomycin, 7.04 ± 5.97% *vs* 0.26 ± 0.10%. The proportions of Fluo-3-binding cells in salvaged samples were much higher than the corresponding proportions of double-positive cells at all ionomycin concentrations tested.

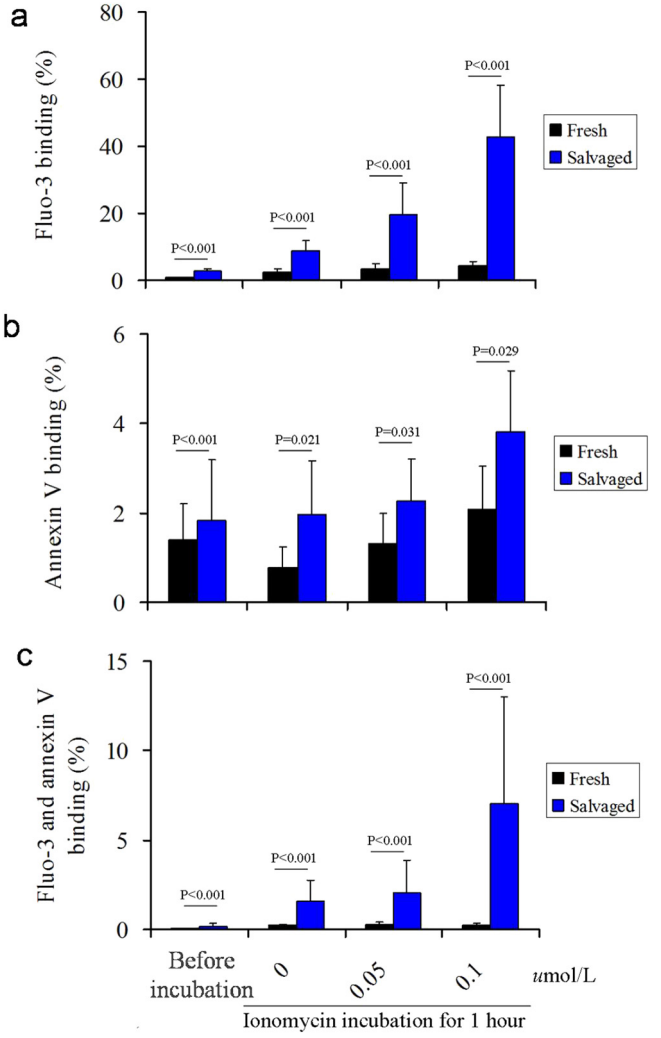

Fig. 3. Effects of incubating salvaged and fresh RBCs from the same patient with the Ca^{2+} ionophore ionomycin. Salvaged and fresh RBCs from each patient were exposed to ionomycin in culture medium containing 1.0 mmol/L Ca^{2+} for 1 h in a 37 °C water bath on a shaker. Percentages of (a) Fluo-3-binding cells, (b) Annexin V-binding cells, and (c) Fluo-3- and Annexin V-binding cells.

The proportion of Annexin V-binding RBCs did not significantly increase with increasing ionomycin concentration in fresh or salvaged samples (Fig. 3b).

3.3. RBC exposure to mannitol-adenine-phosphate (MAP) solution: IBS-induced energy depletion in RBCs triggers Ca^{2+} entry

IBS may induce energy exhaustion in RBCs [16], which inhibits pumping of Ca^{2+} out of the cell by plasma membrane Ca^{2+} ATPase [14]. Therefore we examined whether promoting energy generation can promote Ca^{2+} efflux and therefore avoid the high intracellular Ca^{2+} concentrations associated with RBC damage.

To test this hypothesis, salvaged RBCs were incubated in saline or MAP solution. After 24-h incubation in normal saline, concentrations of each type of adenine nucleotide decreased (Fig. 4a–c), as did the total adenine nucleotide concentration (Fig. 4d). Adenine nucleotide concentrations were higher after incubation in MAP than after incubation in saline (all $P < 0.01$).

The proportion of Fluo-3-binding cells increased with duration of incubation in saline or MAP. This proportion was significantly lower in MAP solution than in saline after incubation for 24 h (3.57 ± 1.44% *vs* 9.64 ± 3.13%, $P < 0.001$, Fig. 5a). Similar results were observed for the proportion of cells positive for both Fluo-3 and Annexin V (1.18 ± 0.35% *vs* 1.47 ± 0.37%, $P = 0.047$, Fig. 5c), and for the proportion of spherocytes after incubation for 24 h (2.45 ± 1.11% *vs* 7.71 ± 2.01%, $P < 0.001$, Fig. 5d).

In contrast, the proportion of Annexin V-binding cells did not differ significantly between samples incubated for 24 h in MAP solution or saline (Fig. 5b).

4. Discussion

Our results suggest that IBS shortens RBC lifespan by inducing morphological changes that transform biconcave RBCs into spherocytes, as well as by exposing PS on the RBC surface, both of which would lead to RBC entrapment in the spleen [19] or engulfment by phagocytes [11,20]. The main cause of morphological changes may be IBS-induced energy exhaustion and Ca^{2+} entry, which can be partially reversed by promoting energy generation. IBS-induced exposure of PS on the RBC surface may occur *via* Ca^{2+}-dependent mechanisms, which can be partially reversed by promoting energy generation, as well as *via* Ca^{2+}-independent mechanisms. These findings may help explain how IBS shortens RBC lifespan and inspire strategies to prolong it and thereby reduce the need for postoperative allogeneic RBC transfusion.

Low intracellular Ca^{2+} levels are important for proper cell volume and rheological properties, redox state, and cell clearance [12]. Increased Ca^{2+} concentration in RBCs may promote cytoskeleton degradation [21] and irreversibly convert the biconcave cells into spheroechinocytes and spherocytes [22–24]. At the same time, alterations in cytoskeletal proteins connecting the spectrin network to the membrane induce cell stiffening. These stiff cells are more likely to be retained in the spleen because of their decreased deformability, regardless of whether PS is exposed on the surface. Proportions of Fluo-3-binding RBCs and spherocytes – all of which were Fluo-3-positive – were much higher in salvaged samples than in fresh samples, and these proportions increased with ionomycin treatment in a dose-dependent manner. These results suggest that salvaged RBCs are prone to Ca^{2+} overload and to the resulting morphological changes that trigger their clearance from the body. These trends are likely to increase with time: proportions of Fluo-3-binding cells and spherocytes in salvaged samples increased with length of incubation in plasma, and these proportions were much higher than proportions of Annexin V-binding cells at all time points.

The plasma membrane Ca^{2+} ATPase in RBCs is the sole mechanism for maintaining low intracellular Ca^{2+} concentration in cytoplasm [25,26], and IBS-induced energy depletion inhibits ATP-dependent efflux of Ca^{2+}. Therefore we examined whether promoting energy generation in RBCs through incubation in MAP solution might reduce Ca^{2+} levels in cytoplasm and mitigate IBS-induced damage of RBCs. Indeed, incubation in MAP solution reduced the proportion of Fluo3-positive cells and the proportion of spherocytes.

Our results are consistent with the idea that IBS induces an increase in intracellular Ca^{2+} concentration in RBCs. One consequence is morphological changes leading to spherocytes, which are engulfed by phagocytes. Another consequence may be activation of a Ca^{2+}-sensitive scramblase that exposes PS on the surface of RBCs [27,28], leading again to phagocyte engulfment [10,20,29]. Consistent with this idea, we found a significantly higher proportion of Annexin V-binding cells in salvaged samples than in fresh samples from the same patient. To test more directly whether IBS increases PS exposure on the RBC surface, we measured proportions of Fluo-3- and Annexin V-positive cells after treatment with ionomycin or MAP solution. The proportion of double-positive cells in salvaged samples was many-fold higher after exposure to ionomycin, and this increase was reversed by MAP. These results suggest that cytosolic Ca^{2+} accumulation is important for triggering PS exposure.

In this way, our results suggest at least two mechanisms by which

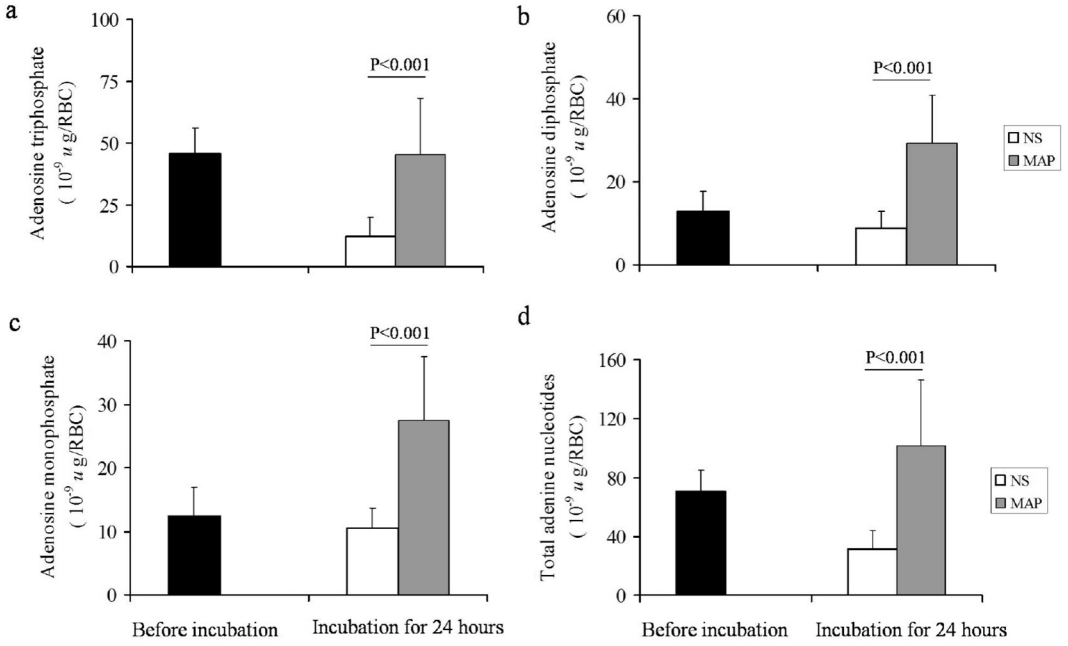

Fig. 4. Concentrations of adenosine triphosphate (a), adenosine diphosphate (b), adenosine monophosphate (c) and total adenine nucleotides (d) in salvaged RBCs from the same patient before and after 24 h incubation with normal saline (NS) or mannitol-adenine-phosphate (MAP) solution.

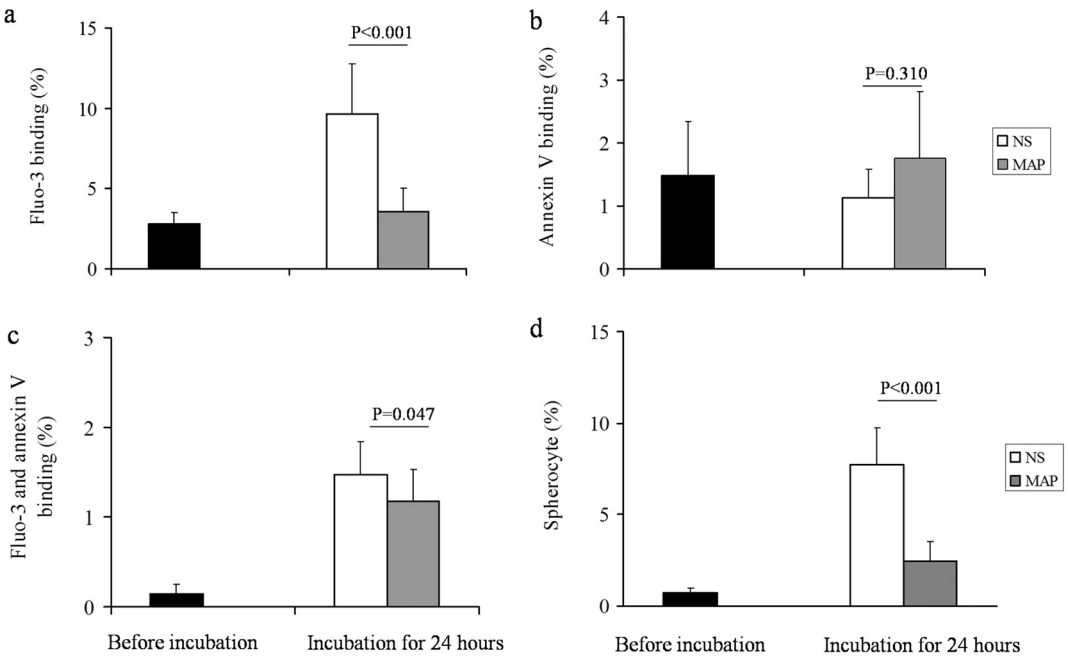

Fig. 5. Percentage of salvaged RBCs from the same patient after 24-h incubation with normal saline (NS) or mannitol-adenine-phosphate (MAP) solution. (a) Fluo-3-binding cells, (b) Annexin V-binding cells, (c) Fluo-3- and Annexin V-binding cells, (d) spherocytes. This experiment was carried out on a group of patients different from the group in Fig. 2.

IBS may trigger phagocyte engulfment of salvaged RBCs: morphological deformation and PS exposure, both of which depend on energy depletion and elevated intracellular Ca^{2+} concentration. A third mechanism, independent of Ca^{2+} entry and ATP depletion, is possible. We observed that the population of Annexin V-positive, Fluo-3-negative cells (Zone 1 in Fig. 1), which was larger in salvaged samples than in fresh ones, did not increase in the presence of ionomycin or decrease in the presence of MAP. These results suggest that IBS may induce PS exposure *via* Ca^{2+}- and ATP-independent pathway(s). Future work should examine this possibility.

In contrast to our results, another study [30] reported that salvaged RBCs showed better membrane deformability than fresh RBCs. However, patients in that study received 527–703 mL of salvaged blood per person, which is only about 10% of total blood volume in the body. This suggests that 90% of the RBCs in blood samples from patients who received salvaged blood were fresh, and the actual proportion may have been even higher because salvaged RBCs should be eliminated from the body with time. Therefore, our experiments in the present study may provide a more reliable characterization of salvaged RBCs. Our previous work showed that membrane deformability of salvaged RBCs worsened during 48-h incubation [16].

We emphasize that our conclusion that salvaged cells are most likely

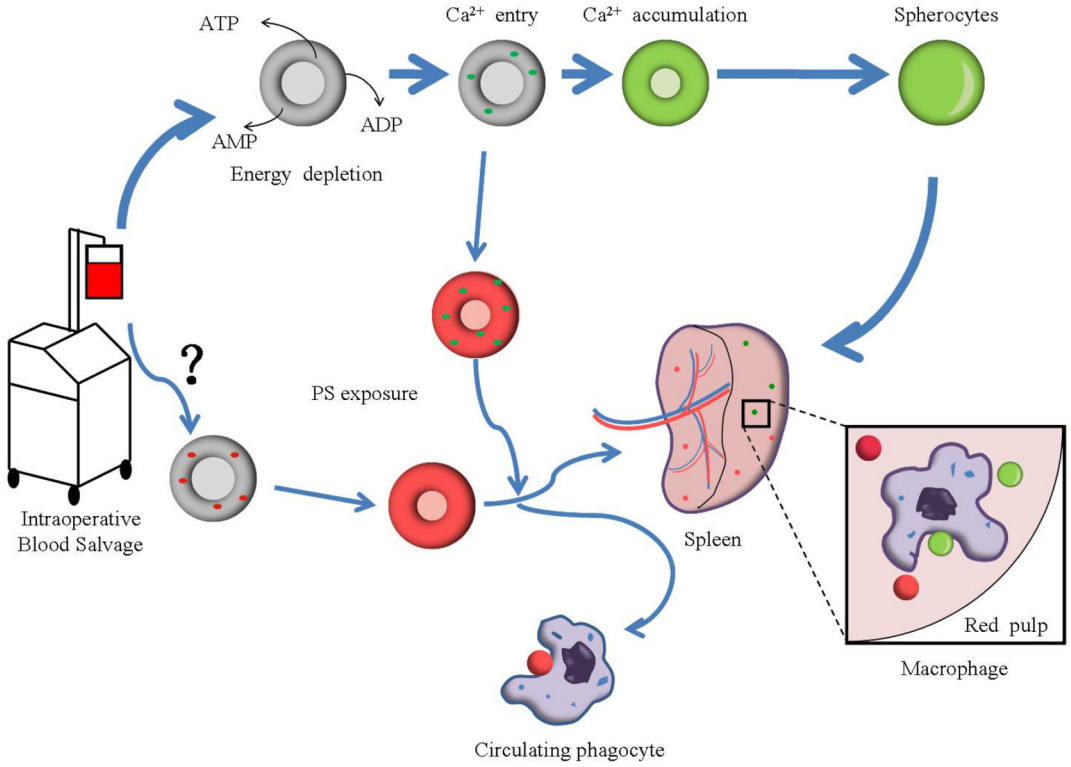

Fig. 6. Mechanisms by which IBS may compromise the lifespan of RBCs. During salvage, energy in RBCs is depleted, which induces Ca^{2+} entry, leading in turn to significant proteolysis of membrane cytoskeletal proteins. Accumulation of Ca^{2+} inside RBCs irreversibly converts them from biconcave discs to spherocytes. Meanwhile, PS is exposed on the RBC membrane through mechanisms that are Ca^{2+}-dependent and -independent. Spherocytes and cells with exposed PS are eliminated by phagocytes.

engulfed by phagocytes comes from *in vitro* data showing that phagocytes recognize PS on the RBC membrane and engulf the cells [11,20], and that IBS patients contain higher levels of 235a-positive phagocytes in circulation than non-IBS patients [10]. RBCs that lose surface area and become spherocytes have been shown *in vitro* to be trapped in the spleen [19]. Our hypothesis should be thoroughly verified in appropriate studies *in vivo*.

5. Conclusion

In conclusion, the present study provides evidence that IBS may injure RBCs by inducing postponed morphological changes or by exposing PS on the RBC surface (Fig. 6). Morphological changes may be due primarily to intracellular Ca^{2+} accumulation reflecting low ATP levels available to drive Ca^{2+} efflux. PS exposure can occur *via* mechanisms dependent and independent of Ca^{2+} entry. Our observations suggest that salvaged RBCs can be damaged and show characteristics similar to those of RBCs that have aged during storage, *i.e.* elevated Ca^{2+} concentration, PS exposure, and cell deformation [11,31]. Our results suggest that promoting energy generation may be an effective strategy to prolong the lifespan of salvaged RBCs. This may have implications for blood treatment procedures that may help IBS reduce the need for blood transfusions over several postoperative days.

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