Original Article
Effects of red blood cell supernatants on hypoxia/reoxygenation injury in H9C2 cells

Fengyan Fan[1,2], Liping Sun[1], Dongqing Zhang[1], Liguo Zhu[1], Shuying Wang[1], Deqing Wang[1]

[1]The Department of Blood Transfusion, The PLA General Hospital, Beijing 100850, China; [2]Department of Blood Transfusion, Air Force General Hospital, PLA, Beijing 100142, China

Abstract: Background: During storage, Red Blood Cell (RBC) undergo changes in morphology, function, and metabolism. Although multiple studies found that longer duration of storage was associated with increased risks of adverse clinical outcomes, especially in critically ill patients, this relationship has not been fully explored. Moreover, the effects of RBC supernatants on cell models simulating critically ill patients have been studied rarely. This study aimed to investigate the possible cytotoxic effects of RBC supernatants on H9C2 cells subjected to Hypoxia/Reoxygenation (H/R) injury. Method: Hypoxia-treated H9C2 cells were treated with RBC supernatants that had been stored for 0 or 35 days. Cells were reoxygenated and incubated for 2 hours. We analyzed cell viability using the Cell Counting Kit-8 (CCK-8) assay, apoptosis using Acridine Orange/Ethidium Bromide (AO/EB) staining and an annexin V-FITC/PI assay, mitochondrial membrane potential (MMP) using a JC-1 staining assay, and ATP levels using the Enzy light ATP Assay kit. Results: H/R-injured H9C2 cells showed significantly reduced cell viability, increased apoptosis, and decreased MMP compared to normally cultured cells. There were no significant differences between H9C6 cells treated with DMEM vs. RBC supernatants stored for 0 or 35 days in terms of cell viability, apoptosis, MMP, or ATP level, nor were there any significant differences between the 0- and 35-days groups. Conclusion: RBC supernatants stored for 0 or 35 days had no effects on H/R-injured H9C2 cells in terms of cell viability, apoptosis, MMP, or ATP level.

Keywords: Blood transfusion, red blood cells, metabolites, cytotoxicity, H9C2 cell, hypoxia/reoxygenation injury, apoptosis

Introduction

Red blood cell (RBC) transfusion therapy is a common treatment in modern medical practice, and millions of transfusions are given annually worldwide [1, 2]. In most countries, RBC unites have a shelf life of up to 35 or 42 days. During storage, red blood cells undergo changes in morphology, function, and metabolism, resulting in unique metabolomic and proteomic profiles for the RBC supernatants [3, 4]. Transfusion of RBC supernatants may lead to clinically adverse effects for recipients from a metabolomics standpoint [3]. Multiple studies found that longer storage of RBC units was associated with increased risks of infection, renal dysfunction, respiratory failure, multiple organ dysfunction syndrome, deep vein thrombosis, and mortality, especially in critically ill patients [5-7]. Several studies have tested the impact of stored RBC supernatants on cardiovascular function, for which supernatants collected from older RBC induced more hypertension after infusion into rats [8]. Perioperative transfusion of washed RBCs and platelets during cardiac surgery reduced postoperative inflammation and the number of postoperative transfusions [2]. Nevertheless, several recent large prospective randomized-controlled trials have examined the harmful effects of transfusing long-term stored RBCs, and found no statistical significant difference [9-11]. The relationship between RBC storage time and clinical events remains unclear. Hence, there is a major interest in whether RBC supernatants stored for longer periods can increase I/R-induced myocardial injury.

The H9C2 cell line is a well-characterized and widely used cell line to study I/R-induced myocardial injury and cardiotoxicity of new drugs. To date, no study has focused specifically on measuring or modeling RBC supernatants-related cytotoxicity after transfusion. RBC stor-
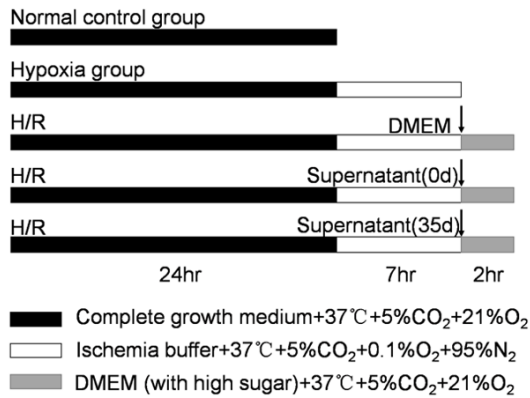
Figure 1. Schematic for the H/R model.

age under standard conditions is associated with metabolomic and proteomic changes of the RBC supernatants, which we hypothesized could increase I/R-induced myocardial injury. In this study, we examined the *in-vitro* cardiotoxicity of RBC supernatants on H9C2 cells, which are sensitive to toxicants. To obtain more obvious results, we used an *in-vitro* H/R model to simulate severe damage.

Materials and methods

Study samples

Five units of pre-stored leuko-reduced RBCs suspended in an additive solution (NaCl-phosphate-adenine-dextrose-mannitol-citrate, MAP) in a PVC-DEHP container (NIGALE, China) were received from Chinese PLA Center for Clinical Blood Transfusion Medicine. These blood units had been donated by five healthy male donors (age: 25-31 years). Suspended RBC units were split into 100-mL aliquots in replicate 180-mLtransfer bags on the day of collection. All bags were stored at 2 to 6°C under blood bank conditions. Supernatant was isolated by centrifugation at 1500 *g* for 10 min. Supernatant was taken from each divided RBC unit on days 0 and 35 of storage and stored at -80°C until use. Studies were performed on the supernatant.

Cell culture and experimental protocols

H9C2 cells (China Infrastructure of Cell Line Resource, Beijing, China) were cultured in DMEM supplemented with 10% fetal bovine serum (both from Invitrogen, Carlsbad, CA) in a humidified atmosphere with 5% CO_2 at 37°C.

To induce ischemia, cells were placed in an ischemic buffer (137 mM NaCl, 12 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 20 mM HEPES, 20 mM 2-deoxy-d-glucose, pH 6.2) [12] at 37°C in an incubator (Thermo 3131, Thermo Fisher Scientific, Waltham, MA) that was maintained with 0.1% O_2, 5% CO_2, and 95% N_2 for 7 hours. Subsequently, cells were re-oxygenated for 2 hours by incubation in normal oxygen conditions in glucose-containing, serum-free DMEM [13]. Before re-oxygenation, hypoxic cells were treated with serum-free DMEM or with RBC supernatants that had been stored for 0 or 35 days.

Experimental groups were as follows: i) normoxia control group; ii) hypoxia group; iii) H/R control group; and iv) treatment groups (0d 35d) (**Figure 1**). There were five replicates in each group. Samples were added to the plating media for H9C2 cells at a final volume of 17%, simulating transfusion of a 60-kg adult with 2000 mL of SRBCs. At the end of the treatments, the cell cultures were photographed under a phase-contrast inverted microscope (Olympus IX51, Japan). All of these experiments were conducted independently in triplicate.

Cell viability assessment

Cell viability was assessed by using Cell Counting Kit-8 (CCK-8; DOJINDO, Japan). CCK-8 solution (10 μL) was added to each well, and the plate was incubated at 37°C for 1.5 hours. Absorbance was measured at 450 nm by using the VICTOR™ X2 Multilabel Plate reader (PerkinElmer, Inc., Waltham, MA).

Acridine orange/ethidium bromide (AO/EB) double-staining

Plasma membrane permeability of H9C2 cells was measured by AO/EB double-staining. H9C2 cells cultured in a 24-well culture plate were treated as described above. Cells were treated with AO/EB (100 μg/mL Sigma-Aldrich) solution for 1 min, and the cell suspension was observed under a fluorescence microscope (Olympus IX51, Japan). H9C2 cells with red or orange nuclei were designated as apoptotic, whereas cells with green nuclei were designated as nonapoptotic. At least 300 cells from several random fields were counted in each group. The apoptotic ratio (%) was calculated as

the number of apoptotic cells divided by the total number of cells (apoptotic + nonapoptotic), multiplied by 100% [14].

Flow cytometry (FCM) using annexin V/PI staining

Apoptosis was assessed by using the Annexin V-FITC kit (MACS, Germany), according to the manufacturer's protocol. After treatment, 10^6 cells were washed and resuspended in binding buffer. A total volume of 10 µL of Annexin V-FITC was added and incubated in the dark for 15 min at room temperature. Cells were washed twice with binding buffer and then resuspended in binding buffer. 5 µL aliquot of propidium iodide (PI) solution was added, and the apoptotic cells were identified immediately by FCM (Beckman Coulter, USA).

Detection of mitochondrial membrane potential (MMP)

MMP changes were measured using the MMP assay and JC-1 kits (Beyotime, China) according to the manufacturer's instructions. Under normal condition, JC-1 can accumulate in the membrane of mitochondria and the mitochondrial membrane showed red fluorescence. When MMP is lost, red fluorescence decreases and green fluorescence increases. The intensity ratio of red to green fluorescence can indicate the change in MMP. JC-1 working solution was added to each well. Samples were incubated in a 5% CO_2 incubator at 37°C in the dark for 20 min. After washing twice with a buffer solution, cells were analyzed by FCM (Beckman Coulter, USA) [15].

Measurement of ATP levels

ATP levels in cells were determined by the Enzylight ATP Assay kit (BioAssay, Systems, Hayward, CA), according to the manufacturer's instructions [16]. H9C2 cells were plated on white 96-well plates, incubated for 24 h, and subjected to H/R treatment. For each 96-well plate, 95 µL of assay buffer was mixed with 1 µL of substrate and 1 µL of ATP enzyme. Then, 90 µL of reconstituted reagent was added to each well, and the plate was mixed by tapping. Luminescence was read on a luminometer (BioTek Instrument, Inc., Winooski, VT) within 1 min after adding reconstituted reagent. Levels for the unknown samples were determined by using an ATP standard curve.

Statistical analysis

Reported data are expressed as the mean ± SD of at least three independent experiments. Statistical analyses were performed using SPSS statistical software V.18 (SPSS Inc., USA). Statistical comparisons between two groups were made by the two-tailed Student's *t*-test. Comparisons among DMEM control group, 0-day and 35-day storage groups were made by one-way ANOVA. Calculations were done in Graph Pad Prism version 5 (GraphPad Software, San Diego, CA) and Excel 2013 (Microsoft). Statistical significance was defined as p < 0.05.

Results

Cell morphology and viability

Seven hours after hypoxia injury, the number of dead cells was increased under the optical microscope. After reoxygenation for 2 hours, the number of dead cells increased further. However, the 0-day and 35-day storage groups showed no significant differences compared to the DMEM control group. The shape of live cells changed from long fusiform to polygonal (**Figure 2**).

Results of the CCK-8 assay (**Figure 2F**) showed that hypoxia and, especially, reoxygenation caused marked reductions in the percentage of viable H9C2 cells (53% and 42%, respectively, compared to 99% for normal controls; p < 0.01). Addition of RBC supernatants that had been stored for 0 or 35 days at 17% of the final volume immediately before reoxygenation had no effect on cell viability after reoxygenation (36.6% and 37.4% viability; p > 0.05). Addition of RBC supernatants stored for longer periods did not cause a significant decrease of H9C2 viability.

Apoptosis

Apoptosis was confirmed by the AO/EB double-staining assay. AO/EB double-stained normal control H9C2 cells blue. H/R-injured cells appeared as bright orange and yellow fragmented cells and were designated as apoptotic (**Figure 3A**). Hypoxia and, especially, reoxygenation markedly increased the apoptosis ratio of H9C2 cells to 16.40% and 49.60%, respectively, compared to controls (0.20%; p < 0.01). H9C2 cells treated with RBC superna-
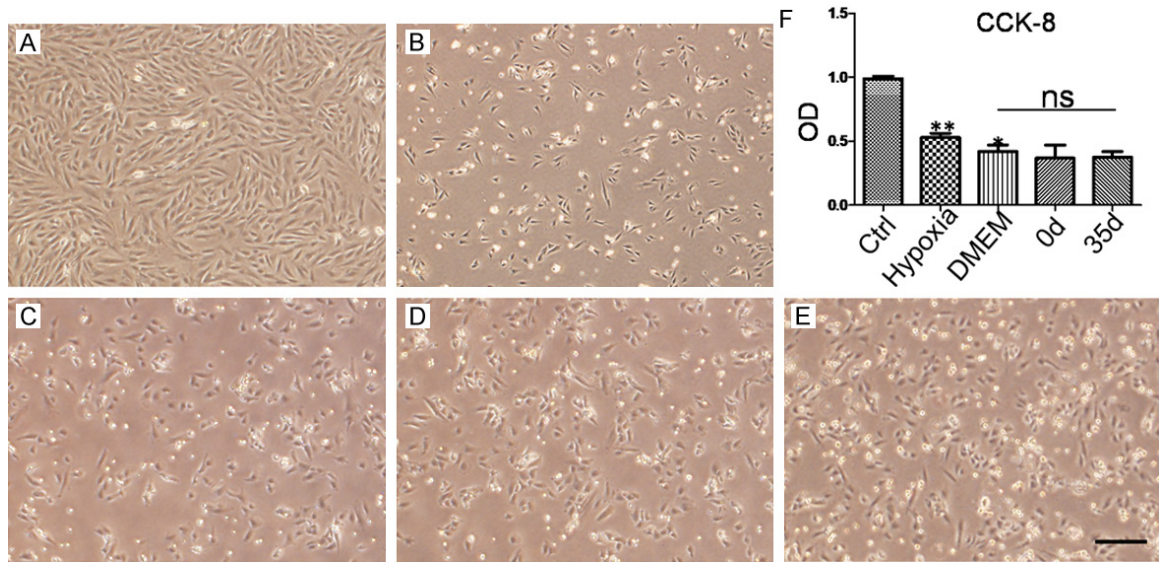
Figure 2. Morphological changes of H9C2 cells under light microscopy. A. Normal control group. B. Hypoxia group. C. H/R DMEM control group. D. 0-day storage group. E. 35-day storage group. Scale bar = 10 μm. F. CCK-8 assay. *p < 0.05 vs. hypoxia group, **p < 0.01 vs. control, ns: no statistical difference.
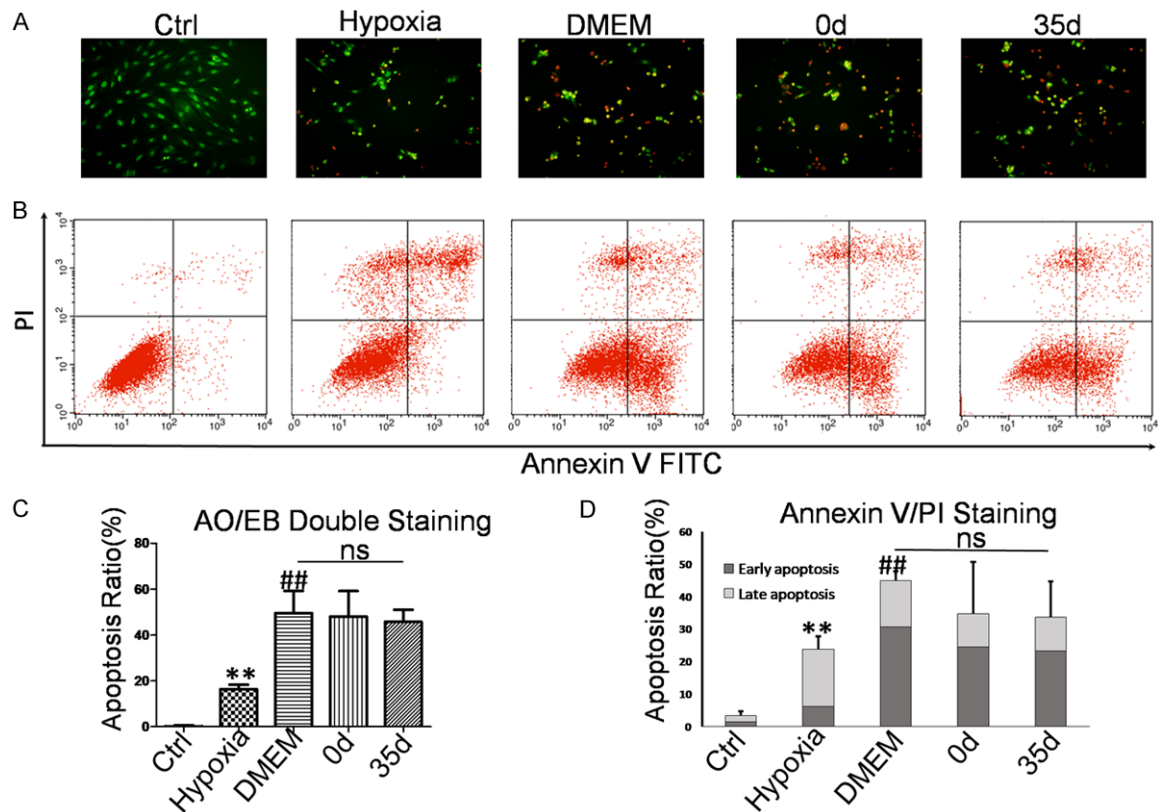

Figure 3. Apoptosis of H9C2 cells assessed by AO/EB double-staining (A) and FCM with annexin V/PI staining (B). (A) AO/EB double staining. ×100 magnification. (B) FCM using annexin V/PI staining. Bottom-left, bottom-right, and top-right quadrants reflect percentages of normal cells, early apoptotic cells, and late apoptotic cells, respectively. (C) Apoptotic ratio calculated as the percentage of the total number of cells. (D) Apoptotic ratio determined by FCM with annexin V/PI staining. Numerical data are presented as the mean ± SD. **p < 0.01 vs. control, ##p < 0.01 vs. hypoxia group, ns: no statistical difference.
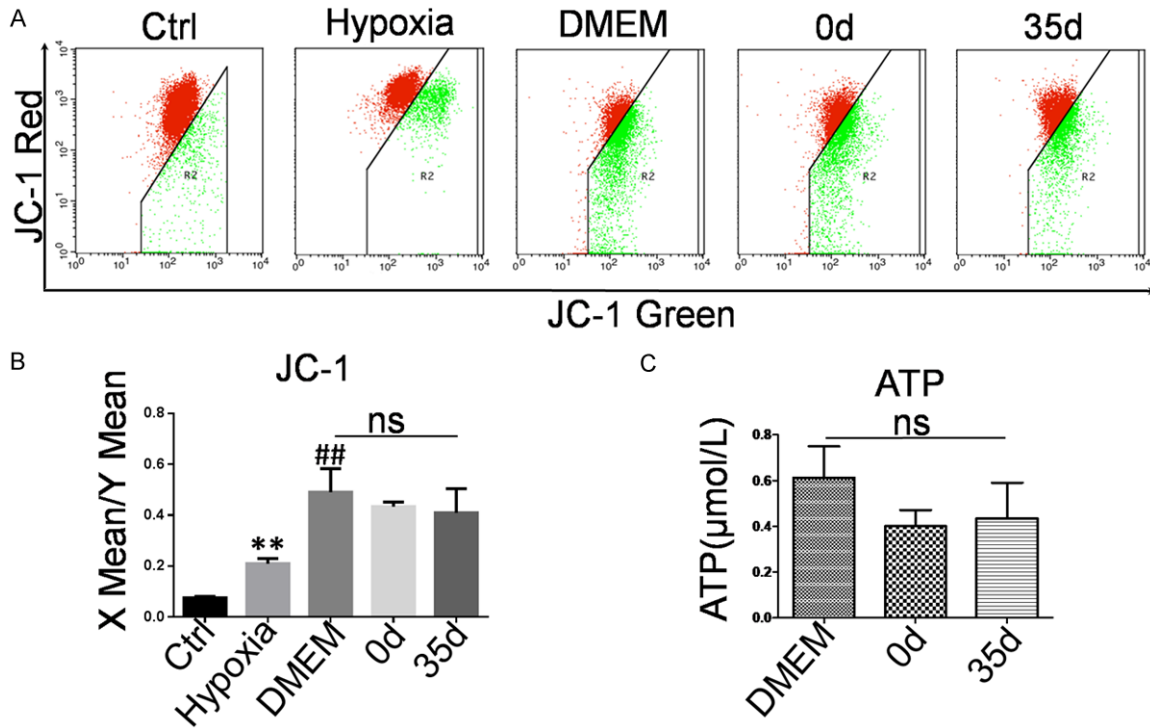
Figure 4. Effects of JC-1 staining and ATP levels. A. Effects of JC-1 staining. R2 regions indicate cell populations with reduced MMP. Other regions indicate cell populations with normal MMP. B. Ratio of X mean toY mean. C. ATP levels. Numerical data are presented as the mean ± SD. **$p < 0.01$ vs. control, ##$p < 0.01$ vs. hypoxia group, ns: no statistical difference.

tants stored for 0 or 35 days showed no significant difference from the DMEM control group (48.00% and 45.00% viable cells, respectively, compared to 49.60% for controls; $P > 0.05$) (**Figure 3C**).

Apoptosis was further assessed by using FCM with annexin V/PI staining. Hypoxia- and, especially, reoxygenation-injured H9C2 cells exhibited increased phosphatidylserine externalization compared to normal control cells (**Figure 3B**). The apoptosis ratio of early and late stages of cells increased significantly from 1.47% (control) to 6.08% after 7 h of hypoxia. After reoxygenation, this ratio increased to 30.70% for cells treated with DMEM, 24.39% for cells treated with 0-day RBC supernatants, and 23.19% for cells treated with 35-day RBC supernatants ($p > 0.05$; **Figure 3D**).

MMP and ATP levels

The membrane-permeable JC-1 dye is widely used to assess MMP in apoptosis studies. We observed a significant increase in MMP of H/R-treated H9C2 cells (control: 0.08, hypoxia:

0.21, reoxygenation: 0.49; $p < 0.05$). We found no significant difference in MMP or ATP levels between DMEM-treated cells (MMP: 0.49, ATP: 0.44 µmol/L), 0-day RBC supernatants-treated cells (MMP: 0.43, ATP: 0.61 µmol/L), and 35-day RBC supernatants-treated cells (MMP: 0.41, ATP: 0.40 µmol/L) (**Figure 4B**, **4C**).

Discussion

Whether the duration of RBC storage is associated with increased risks of clinically adverse events remains unclear. Alexander et al. summarized the results of randomized trials evaluating the effect of the age of transfused RBCs and concluded that existing evidence provides no support for changing practices towards fresher RBC transfusions [17]. However, the outcomes of clinical trials are affected by many factors, including the heterogeneous distribution of blood samples (fresh and old) and differences in the preparation of blood products (e.g., leukocyte depletion steps, storage media, irradiation guidelines, etc.), study design, sample population, and statistical approach [18, 19]. In this study, we investigated the possible

cytotoxic effects of RBC supernatants on H9C2 cells subjected to H/R injury. Consistent with recent clinical results, we found that there was no significant difference in terms of cell viability, apoptosis, MMP, or ATP level between H/R-injured cells when treated with DMEM compared to RBC supernatants that had been stored for 0 or 35 days.

The H9C2 line of embryonic rat cardiomyocytes is commonly used for in-vitro analyses of cardiotoxicity, apoptosis, and necrosis [20, 21]. The H/R model of H9C2 cells simulates heart ischemia/reperfusion injury, which involves nitro-oxidative stress and inflammation. To simulate restoration of the oxygen supply by blood transfusion, we added RBC supernatants just before reoxygenation. If the RBC supernatants had adverse effects on H9C2 cells, then the results would have been more striking on the ischemia-reperfusion injury model. Most studies of RBC storage time are performed in patient groups receiving, on average, one to four RBC unit transfusions [22, 23]. Here, we looked at the observational results in more heavily transfused patient populations, simulating a typical blood transfusion volume of 2000 mL for a body weight of 60 kg (17% v:v).

Previous research on the influences of RBC supernatants focused on the immunologic functions of immunocytes [24, 25], whereas no study to date has looked at cytotoxicity of myocardial cells in vitro. Various methods, with distinct sensitivities, specificities, and principles, are commonly used to assess cell injury in vitro. The CCK-8 assay is a sensitive colorimetric assay for determining the number of viable cells in cell proliferative and cytotoxicity experiments. The amount of formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells. We measured the plasma membrane permeability of H9C2 cells by AO/EB fluorescent double-staining, which can be used to identify cell membrane changes during apoptosis [26]. Phosphatidylserine redistribution from the inner to the outer leaflet is an early and widespread event during apoptosis. The annexin V/PI staining assay can be used to detect the early phases of apoptosis sensitively and easily before the loss of cell membrane integrity [27]. The decrease in $\Delta\Psi m$ is a sign of early apoptosis. JC-1 is a membrane-permeable dye that is widely used to assess MMP in apoptosis stud-

ies. In this article, we used different methods to assess the injury of H9C2 cells, including cell morphology, cell viability, apoptosis, MMP, and ATP level. Our findings confirmed that RBC supernatants had no effects on H9C2 cells subjected to H/R injury.

Our study has some limitations. Firstly, embryonic rat cardiomyocytes (H9C2 cells) were used for the in-vitro study of human RBC supernatants. This choice may limit translation of our findings to the clinic [28]. Secondly, in the period of RBC storage, changes occur in the levels of potassium, sodium, chloride, and calcium ions [29, 30]. H9C2 cells do not beat rhythmically, which is easily affected by metal ion levels. It is possible that our study did not fully consider the toxic effects of RBC supernatants. Future studies should be applied to human and beating cardiomyocytes, such as human induced pluripotent stem cell-derived cardiomyocytes, to evaluate the cytotoxicity of RBC supernatants in vitro.

In conclusion, we found that supernatants from leuko-reduced RBC units stored for 0 and 35 days had no effects on H/R-injured H9C2 cells in terms of cell viability, apoptosis, MMP, or ATP level. This in-vitro result is consistent with recent clinical results that there is currently no evidence to support changing practices towards fresher RBC transfusions [17].

Acknowledgements

Disclosure of conflict of interest

All authors declared there were no conflict of interests involved.

Address correspondence to: Deqing Wang, The Department of Blood Transfusion, The PLA General Hospital, 1, No. 28, Fuxing Rd, Beijing 100850, China. Tel: +86-010-66936508; Fax: +86-010-66936508; E-mail: deqingw@vip.sina.com

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