Original Paper

Induction of Pluripotent Stem Cell-Derived Cardiomyocyte Toxicity by Supernatant of Long Term-Stored Red Blood Cells *in Vitro*

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Key Words

Cardiotoxicity • Human-induced pluripotent stem cell-derived cardiomyocytes • Suspended red blood cells • Real-time cell analyzing

Abstract

Background/Aims: Preserved red blood cells (RBCs) *in vitro* undergo a series of morphological, functional and metabolic changes during storage. RBC metabolites accumulate over time during storage, the toxicity of the supernatants of RBCs (SSRBCs) on tissue cells is largely unknown. Here, we aimed to study cardiomyocyte toxicity by supernatant of long term-stored RBCs *in vitro* and to discover elements involved in the mechanism. **Methods:** Using human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) and real-time cell analyzing (RTCA), we analyzed the cardiotoxicity of d0, d14 and d35 SSRBCs. To analyze the cardiotoxicity of potassium (K) and lactic acid (LA) in SSRBCs, solutions containing the same concentrations of K and LA were respectively prepared and co-cultured with hiPS-CMs. Immunofluorescence and Gene Expression Array of hiPS-CMs were performed to evaluate the effects of d35 K and d35 SSRBCs. **Results:** The beating of hiPS-CM was stopped by d14, d35 SSRBCs, or d35 K solution. Beating resumed within 48 hours in the presence of d14 SSRBC or d35 K but not d35 SSRBC; d0, d14 and d35 LA solution had no effect on beating patterns. At 48h after treatment, the immunofluorescence results showed that the integrity of the filament and sarcomere were intact. Gene Expression Array results found 14 differentially expressed genes which were likely to play an important role in the cytotoxic effect. **Conclusion:** Our results demonstrated cardiomyocyte toxicity by long term-stored SSRBCs *in vitro*. Besides high K-induced cardiotoxicity, there must be other unknown components in long term-stored SSRBCs that are cytotoxic to hiPS-CMs.

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Introduction

Red blood cells (RBCs) transfusion therapy is a common treatment in modern medical practice. Approximately 100 million units of RBCs are transfused annually worldwide [1]. In

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most countries, suspended RBCs (SRBCs) have a shelf-life of up to 35 or 42 days. However, preserved RBCs *in vitro* undergo a series of morphological, functional and metabolic changes during storage [2-5]. Extended storage periods trigger eryptosis, the suicidal erythrocyte death [6]. Since RBC metabolites can accumulate over time during storage and lead to great changes in the metabolomics and proteomics of the supernatants of RBCs (SSRBCs), their effects on tissue cells are uncertain [7]. Some studies have reported that SSRBCs have immunosuppressive effects on immune cells [8, 9], but none of these studies examined specifically the cytotoxicity caused by SSRBCs.

Although a large number of retrospective and prospective clinical studies have studied the relation between duration of RBCs storage and adverse events of transfused patients, whether “fresh” blood is better than “old” blood is currently still a controversial topic [10]. Recently, several studies have shown that longer storage of RBCs was associated with increased risks of infection, renal dysfunction, respiratory failure, multiple organ dysfunction syndrome, deep vein thrombosis, and mortality. In addition, adverse clinical outcomes were more common especially in critically ill patients [11-13]. A study by Cholette et al. found that transfusion of washed RBCs and platelets during cardiac surgery reduced postoperative inflammation and number of transfusions [14]. However, several recent large prospective randomized-controlled trials (RCTs) have examined the harmful effects of transfusing long-term stored RBCs, and found no statistical significant difference in the in-hospital mortality among a general population of hospitalized patients [15], frequency of 5 major adverse outcomes among premature low-birth-weight infants [16], 90-day mortality among critical care patients [17] and Multiple Organ Dysfunction Score following cardiac surgery [18]. Therefore, whether the duration of RBCs storage is associated with increased risks of clinically adverse events is still unclear [19].

In this study, we examined the cardiotoxicity induced by SSRBCs utilizing human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) to directly evaluate the cytotoxic effects of SSRBCs. The use of hiPS-CMs eliminates the inter-species difference between mice and human, and faithfully reproduces cardiac electrophysiological properties of human cardiomyocytes [20]. Unlike primary rat neonatal cardiomyocytes, the beating activity of hiPS-CMs is more stable and is barely influenced by the seeding density of hiPS-CMs or medium changing. Therefore, hiPS-CMs have gained wide acceptance as a biologically relevant *in vitro* model for drug discovery and cardiotoxicity screens [21, 22]. Compared to cytotoxicity assays on H9C2 cell line, hiPS-CMs are more sensitive for the detection of potential cardiac side effects which can be indicated by the beating patterns of CMs [23]. The real-time cell analyzing (RTCA) Cardio system is a non-invasive, impedance-based biosensor system that can monitor CMs growth and beating status simultaneously in a drug-sensitive manner [24, 25].

In the present study, we examined the cardiotoxicity of SSRBCs on human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) which are sensitive to toxicants. The beating of hiPS-CMs was recorded in real time using the Real-Time Cell Analyzing (RTCA) assay to determine the time-dependent cardiotoxicity of long-term preserved SSRBCs. In addition, we also investigated the mechanism of cardiotoxicity by examining the effects of potassium (K) and lactic acid (LA) in the SSRBCs, which may be the potential toxic components, and gene expression in beating arrested hiPS-CMs through mRNA microarray.

Materials and Methods

Sample collection

We obtained five blood donations from the Chinese PLA Center for Clinical Blood Transfusion Medicine. These blood units had been donated by five healthy male donors (age: 25–31 years). Five RBCs samples suspended in the additive solution NaCl-phosphate-adenine-dextrose-mannitol-citrate (MAP) in a PVC-DEHP container (NIGALE, Sichuan, China) were obtained. All RBC components were leukoreduced using RBC filters (NIGALE, Sichuan, China) and stored at 4°C according to blood bank requirements. Suspended

RBC units were split into three aliquots equally on the day of collection, whose volume was about 100mL. Then supernatant was isolated on d0, d14, and d35 of storage by centrifugation at 1500g for 10min. The obtained supernatants were then stored at -80°C for subsequent use.

Detection of biochemical indicators and preparation of K and LA solutions

Biochemical indicators in the SSRBCs were examined using biochemical analyzer (VITROS 5600, Johnson & Johnson). Data of d0, d14, and d35 SSRBCs were expressed as mean $\pm$ SD. K and LA solution were prepared using KCl (Lot #BCBN5704V, Sigma) and L-(+)-Lactic acid (Lot #SLBM5936V, Sigma) with maintaining medium (CELLAPYBIO, China), respectively. The concentrations of the K and LA solutions were corresponding to that in d0, d14 and d35 SSRBCs respectively. K and LA levels were tested again after adding SSRBCs to the hiPS-CMs maintaining medium (17% final volume).

Cell culture and treatments

HiPS-CMs were a well-validated cell line [26] purchased from CELLAPYBIO (Cat# CA2001106, Beijing, China). HiPS-CMs were thawed following recommended procedures. Cardio E-Plate 96 (ACEA Biosciences, San Diego, USA) was coated with 50 μ L of 1:100 matrigel solution (BD Biosciences, USA) and stored at 4°C overnight. Each well was then seeded with 150 μ L of hiPS-CMs plating medium containing 17,000 cells, and the plate was placed into the xCELLigence System RTCA Cardio instrument (ACEA Biosciences, San Diego, USA). The cells were incubated in a humidified atmosphere at 37°C with 5% CO₂ until the cells reached >80% confluence. Cell culture medium was refreshed every 2 days.

The medium in Cardio E-Plate was replaced with hiPS-CMs maintaining medium 4h before treatment. HiPS-CMs were treated with d0, d14, and d35 SSRBCs or maintaining medium. Positive inotropic compounds amiodarone (Ami) and doxorubicin (Dox) were tested on hiPS-CMs as positive controls (10 μ M). SSRBC samples were added to the wells in hiPS-CMs maintaining medium (17% final volume) to simulate the transfusion of large blood volume. This volumetric relationship is equivalent to the infusion of 2000ml suspended RBCs in a 60 kg adult, which is approximately 80 ml of blood/kg of body weight [9].

Real-time cardiomyocytes analysis

The cardiomyocyte-based biosensor can monitor the cardiomyocyte cell index (CI) and beating status simultaneously. The device, which was placed inside the incubator, was able to switch from any wells on the E-plate 96 to the RTCA Cardio system analyzer for impedance measurement. The CI value can reflect the differences in cell number, level of adhesion, cell morphology and viability. CI values increased gradually and consistently as cells become attached to the electrodes [27]. Beating status of CMs was described through the beating parameters beating rate (BR) and amplitude (A). The normalized cell index (NCI) was the ratio of CI at the time of interest to the CI taken prior to the treatment. The normalized beating rate (NBR) and normalized amplitude (NA) were similarly calculated. CI was recorded every 5 min, and beating status was recorded every 30 min for 48h following SSRBCs treatment.

Evaluation of the cardiotoxicity of K and LA solution

In order to determine whether K and LA in the SSRBCs have cytotoxic effects on hiPS-CMs, the process of cell culture was repeated and the premade LA solution, K solution, and mixture of K and LA solution were co-cultured with hiPS-CMs (17% final volume). Mixture of d0, d14 and d35 SSRBCs samples were simultaneously measured as controls.

Immunofluorescence assay

HiPS-CMs were placed on glass coverslips and treated with d35 K solution, mixed d35 SSRBCs or maintaining medium (17% final volume) while beating steadily. The mixed d35 SSRBCs sample contains a mixture of five d35 SSRBCs samples. At 48h after treatment, the cells were washed in PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. The cells were washed in PBS, blocked and incubated with Troponin T type 2 (TNNT2) or α -actin antibody (1:200 dilution) at 4°C overnight. On the following day, the cells were incubated with fluorescent labelled goat anti-mouse IgG and goat anti-rabbit IgG secondary antibody for 1 h, and the nuclei were then counterstained with DAPI (100 ng/ml) for 10 min. The images were acquired using a laser confocal microscope (Leica TCS SP5 II, Heidelberg, Germany).

RNA isolation and microarray analysis

HiPS-CMs were seeded in a 6-well plate and treated with mixed d35 SSRBCs, d35 K or the maintaining medium (17% final volume) and incubated for 48h. At 24h and 48h after treatment, cells were photographed under a phase contrast inverted microscope (OLYMPUS, Japan). At 48h after treatment, total RNA was also isolated from the cells using RNAiso plus (TaKaRa, Japan) according to the manufacture's protocol. A total of 10 mg purified RNA from each sample was submitted to the Capitalbio Technology (Beijing, China) for labelling and hybridization in the Affymetrix GeneChip PrimeView Human Gene Expression array (Affymetrix).

Microarray data analysis

The R Bioconductor affy package_ENREF_43 RMA (robust multi-array average) function was used to calculate gene expression. The RMA-normalized expression values can be used for sample correlation and differential gene expression analyses. Genes were considered differentially expressed when they displayed significant changes in expression (q-value $\leq$ 5% and fold change $\geq$ 2 or $\leq$ 0.5).

Reverse transcription-PCR (RT-PCR)

In d35 SSRBCs group and naive group, RT-PCR was used to validate the microarray data of differentially expressed genes associated with smooth muscle contraction. Purified RNA was used to synthesize 20 μ l cDNA with the Thermo First cDNA Synthesis Kit (Thermo, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was analyzed by StepOnePlus Real-time PCR System (Applied Biosystems, USA) using the SYBR Green method. Specific primers were used in PCR amplification (Table 1). The mRNA levels of all detected genes were normalized to ACTB.

Statistical analysis

Data were analyzed using the xCELLigence Cardio Software (Roche, Mannheim, Germany). Calculations and further analyses were performed using GraphPad Prism 5 (Graphpad Software, San Diego, CA, USA) or SPSS PASW Statistics v18.0 (Mathworks, Natick, MA, USA). The reported data were expressed as mean $\pm$ SD. Inter-group comparison was performed using one-way ANOVA or Student's t-test. $p < 0.05$ was considered as statistically significant.

Results

Detection of biochemical indicators in SSRBCs

Detection of biochemical indicators showed significantly increased levels of K, Cl, LA and FHb, and reduced levels of Na, pH and Glu in the SSRBCs (Table 2). In particular, the changes in K and LA levels were significant. The final concentrations of K and LA after adding

Table 1. Primer sequences and gene amplicon size accessed by RT-PCR

Gene title	Gene symbol	Sequence (5'-3')	Size
actin	ACTB	F: TGGCGAGAAAACAAGATGAG R: CACCTTCACCGTCCAGTTT	114bp
hemoglobin, beta	HBB	F: CGTGGATGAAGTTGGTGGTG R: AACAGCATCAGGAGTGGACA	106bp
ATP-binding cassette, sub-family G (WHITE), member 1	ABCG1	F: AGGTGGTCTCGCTGATGAAA R: AGGACGTAAAGCTGGTCGAA	109bp
ATP-binding cassette, sub-family A (ABC1), member 1	ABCA1	F: AGCCCTGGATGTACAACGAA R: TTTCTTCCATACAGCGGGT	127bp
TLR4 interactor with leucine-rich repeats	TRIL	F: GCCTAGATTGACCCATGGCT R: GAGGAGACTGCCAGAAAAGC	141bp
sarcomer	SSPN	F: CCCTATTTCGAGCTCACAC R: TGGTACAGTCCGTCACATCC	122bp
calsequestrin 1 (fast-twitch, skeletal muscle)	CASQ1	F: GGCCCATCTCTGTCTCTGTT R: ATTCTCCCTTCTCTGTGGCC	108bp
calponin 1, basic, smooth muscle	CNN1	F: GCCCTGTAGAACTCAACCT R: GGCTCTATGTTGGACCTGCT	118bp
natriuretic peptide receptor 3	NPR3	F: GTTGGTCTCTGGAATCTGA R: ACAGGGTCCAGCAATTGGT	104bp
protein kinase, cAMP-dependent, regulatory, type I, alpha	PRKAR1A	F: CAGCCTTCGAGATGTGAGC R: AATGCCATGGGTCTCTCAGG	108bp
solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	SLC25A4	F: CGAGAGCACGAACGGGCTG R: CCCGCCAGGAAGTCTTTTA	94bp
xin actin-binding repeat containing 2	XIRP2	F: GAGTGGAGGTGAGTCAGAA R: TGGACTGTCTAGTGGCATAG	102bp
ankyrin repeat domain 1 (cardiac muscle)	ANKRD1	F: ACACCTTCAGAGCCTCCCA R: AGGAGAAAAGGGCTGTGA	137bp
nexilin (F actin binding protein)	NEXN	F: GCATGCAGTTTGAACAAGGG R: AGCTCTCTGATCTGTTTGTCT	128bp
triadin	TRDN	F: AAAGCAGCTGTCTCAAGCA R: AGTGGGTCTGGAAGCTTGT	147bp
actin, alpha 1, skeletal muscle	ACTA1	F: CCTGTATGCCAACACGTCA R: GCGGTGATCTTTCTGCAT	81bp

SSRBCs to the hiPS-CMs maintaining medium (17% final volume) are shown in Table 3.

Cardiotoxicity of SSRBCs on hiPS-CMs

The cytotoxic effects of SSRBCs on hiPS-CMs were described by NCI, NA, NBR and beating pattern. As shown in Fig. 1A, d0, d14 and d35 SSRBCs caused a sharp fall in NCI value to 0.4, which was then maintained at a steady level of 0.5. At 48 h, there was no significant difference in NCI value between the three SSRBCs groups, but the NCI values of the three SSRBCs groups were significantly different from that of the naive group. There were no marked changes in NA and NBR values in the d0 group (Figs. 1B and 1C). NA and NBR values in the d14 group were close to zero in the first 20 hours, but were increased after 20 hours. However, there were no significant differences in NA and NBR values between the d0 group, d14 group and naive group at 48h. NA and NBR values in the d35 group at 48h were close to zero and were not analyzed. The 5 individual SSRBCs samples in each of the d14 and d35 group had certain effects on the beating patterns of hiPS-CMs. Both d14 and d35 SSRBCs treatments induced arrest in beating, only the beating patterns in the d14 group were restored at 48h (Fig. 2B). Beating patterns in the d35 group were never restored at 48h (Fig. 2C).

Cardiotoxicity of K and LA solution on hiPS-CMs.

Effect of K and LA solutions on the NCI value and beating pattern of hiPS-CMs showed that the effects of the mixed five d0, d14 and d35 samples on NCI value and beating pattern were consistent with those in Fig. 1A and Fig. 2 (Figs. 3A and 4A). D0, d14 and d35 LA solutions had no effects on NCI value and beating pattern of hiPS-CMs (Figs. 3 and 4). D0 and d14 K solutions had no significant effects on NCI value and beating pattern (Figs. 3 and 4). However, d35 K solution induced an arrest in hiPS-CMs beating and was restored within 48h

Table 2. Biochemical indicators of d0, d14 and d35 SSRBCs (n=5). Data are expressed as mean $\pm$ SD. (*p<0.05, †p<0.01, §p<0.001, vs d14)

Indicator	SSRBCs		
	d0	d14	d35
K (mmol/L)	1.99 $\pm$ 0.34	23.33 $\pm$ 1.91†	40.61 $\pm$ 1.84§
Na (mmol/L)	102.5 $\pm$ 2.5	99.2 $\pm$ 3.6*	87.9 $\pm$ 3.3§
Cl (mmol/L)	70.2 $\pm$ 1.7	72.6 $\pm$ 2.0	75.4 $\pm$ 2.3‡
pH	6.70 $\pm$ 0.05	6.52 $\pm$ 0.05†	6.39 $\pm$ 0.06§
LA (mmol/L)	2.88 $\pm$ 0.23	6.94 $\pm$ 0.75†	14.98 $\pm$ 1.07§
Glu (mmol/L)	30.3 $\pm$ 1.4	28.8 $\pm$ 2.2*	23.5 $\pm$ 3.3‡
FHb (mg/dL)	4.27 $\pm$ 1.77	10.25 $\pm$ 5.32*	22.19 $\pm$ 9.95‡

Table 3. Levels of K and LA after addition of SSRBCs to the hiPS-CMs maintaining medium. Data are expressed as mean $\pm$ SD. (n=5)

Variable	SSRBCs-added maintaining medium (17%)		
	d0	d14	d35
K (mmol/L)	5.50 $\pm$ 0.06	9.13 $\pm$ 0.33	12.07 $\pm$ 0.31
LA (mmol/L)	0.70 $\pm$ 0.04	1.39 $\pm$ 0.13	2.76 $\pm$ 0.18

Fig. 1. Effect of d0, d14 and d35 SSRBCs on the beating parameters of hiPS-CMs. (A) Effect of d0, d14 and d35 SSRBCs on NCI value and data at 48h after treatment. (B) Effect of d0, d14 and d35 SSRBCs on NA value and data at 48h after treatment. (C) Effect of d0, d14 and d35 SSRBCs

on NBR value and data at 48h after treatment. (*p<0.05, †p<0.01, ns p>0.05). Only one-sided SD is shown in left panel in B, C. Ami (amiodarone) and Dox (doxorubicin) were taken as positive inotropic compounds.

(Fig.4D). Unlike the rapid decrease of NCI value (down to 0.5) in the d35 SSRBCs group, the NCI value of the d35 K group decreased slowly to 0.7 at 48h, and the 48h NCI value of the d35 K group was significantly higher than that of the d35 SSRBCs group (Fig.3D). The effects of the mixed K and LA solution on NCI value and beating pattern were consistent with those of the K solution.

Cell morphology

At 24h after treatment, significant cell shrinkage was observed in the hiPS-CMs treated with d35 SSRBCs. At 48h after treatment, significant cell shrinkage was observed in cells treated with d35 K and d35 SSRBCs, and the shrinkage was more prominent in the d35 SSRBCs group (Fig. 5A). The immunofluorescence results also showed cell shrinkage, but the integrity of filament and sarcomere were intact with no nuclear pyknosis detected (Fig. 5B).

Gene expression array

The correlation between the d35 K group, d35 SSRBCs group and naive group were analyzed using the principal component analysis (PCA). There was no separation between the naive and d35K samples. On the contrary, d35 SSRBCs samples segregated distinctly from the naive and d35 K samples (Fig. 6). A total of 54 genes showed increased expression and 86 showed decreased expression (> 2 -fold, $P < 0.05$) in the d35 SSRBCs group compared with the naive group. Fifteen most differentially expressed genes associated with smooth muscle contraction are listed in Table 4. Results of RT-PCR show that expect ANKRD1 the expression of the other 14 genes (HBB, ABCG1, ABCA1, TRIL, SSPN, CASQ1, CNN1, NPR3,

Fig. 2. Changes in hiPS-CMs beating pattern after treatment with d0, d14 and d35 SSRBCs from 5 individuals. (A) Changes in beating pattern after treatment with d0 SSRBCs from 5 individuals. (B) Changes in beating pattern after treatment with d14 SSRBCs from 5 individuals. (C) Changes in beating pattern after treatment with d35 SSRBCs from 5 individuals.

Fig. 3. Effect of K and LA solutions on the NCI value of hiPS-CMs. (A) Effect of d0, d14 and d35 SSRBCs mixtures on the NCI value. (B) Effect of d0 K and d35 SSRBCs, and the shrinkage on NCI value. Ami (amiodarone) and Dox (doxorubicin) were taken as positive inotropic compounds. (C) Effect of d14 K and LA solutions on NCI value. (D) Effect of d35 K and LA solutions on NCI value.

PRKAR1A, SLC25A4, XIRP2, NEXN, TRDN, ACTA1) were consistent to the microarray data (Fig. 7).

Discussion

In past studies, cardiotoxicity was not easily assessed using primary neonatal rat CMs due to the difficulty in purifying cardiomyocytes and the influence of anthropic factors on the counting of beating rate by naked eye under the optical microscope. Using the RTCA Cardio system, we recorded the time-response changes in NCI, NA, NBR, and beating pattern which are not obtainable by naked eyes. Time-dependent cardiotoxicity was examined in the three SSRBCs groups. As the preservation time extended, d0, d14 and d35 SSRBCs demonstrated increasing cytotoxic effects on the hiPS-CMs.

Levels of K and LA in SSRBCs increase substantially during RBC storage due to the metabolism of RBCs, K leakage from the RBCs, and RBC lysis. In our study, the level of K in MAP-suspended RBCs increased from 1.99 to 40.61 mmol/L, and the level of LA increased from 2.88 to 14.98 mmol/L from d0 to d35 of RBCs storage. Similar to our results, the level of K in d35 CPDA-1 whole blood and in d42 SAMG-suspended RBCs were 78.5 mmol/L and 45.3 mmol/L, respectively [28]. Clinical hyperkalemia resulting from RBCs transfusions has been recognized as a transfusion complication for decades. Hyperkalemia can not only lead to cardiac arrests [29], but it also occurs frequently in hospitalized patients and is of particular concern for those who have undergone surgery, with postoperative care provided by clinicians of many disciplines [30]. Clinically significant hyperkalemia can result following the infusion of a number of RBCs units, especially in infants and small children. Whether

Fig. 4. Changes in hiPS-CMs beating pattern after treatment with K and LA solutions. (A) Effect of d0, d14 and d35 SSRBCs mixtures on beating pattern. (B) Effect of d0 K and LA solutions on beating pattern. (C) Effect of d14 K and LA solutions on beating pattern. (D) Effect of d35 K and LA solutions on beating pattern.

Table 4. Fifteen most differentially expressed genes (d35 vs naive) associated with smooth muscle contraction

Gene symbol	Score(d)	q-value(%)	Fold change	Biological function
HBB	26.241	0	170.7	positive regulation of cell death
ABCG1	14.16	0	5.5	ATP catabolic process
ABCA1	17.69	0	3.7	ATP catabolic process
TRIL	6.51	0	3.0	TLR4 signaling pathway
SSPN	-9.79	0	0.5	muscle contraction
CASQ1	-6.05	0	0.5	Calcium regulation in cardiac cells
CNN1	-6.27	0	0.5	regulation of smooth muscle contraction
NPR3	-8.81	0	0.5	negative regulation of adenylate cyclase activity
PRKAR1A	-4.50	0	0.5	regulation of protein phosphorylation
SLC25A4	-4.43	0	0.4	energy reserve metabolic process
XIRP2	-7.92	0	0.3	actin cytoskeleton organization
ANKRD1	-9.82	0	0.3	positive regulation of neuron projection development
NEXN	-21.66	0	0.2	regulation of cytoskeleton organization
TRDN	-6.66	0	0.2	cellular calcium ion homeostasis
ACTA1	-8.20	0	0.2	smooth muscle contraction

Fig. 5. Morphological changes of hiPS-CMs. (A) Morphological changes of hiPS-CMs observed under light microscope. HiPS-CMs were treated for 0, 24 and 48h as indicated. The edges of shrinking cells show enhanced refractivity. HiPS-CMs treated with d35 K for 48h and d35 SSRBCs for 24 and 48h showed significant shrinkage of different degrees. (B) Immunofluorescent staining of TNNT2 (red), nuclei (blue) and α -actin (green) in hiPS-CMs at 48h after treatment as indicated. No fractures of filament and sarcomere, and no nuclear pyknosis were detected.

Fig. 6. Volcano plot of microarray analysis of hiPS-CMs. Red dots represent up-regulated genes and green dots represent down-regulated genes.

Fig. 7. Verification of selected fifteen most differentially expressed genes associated with smooth muscle contraction by RT-PCR (d35 vs naive). Values are represented as the mean $\pm$ SD. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs naive).

hyperlactatemia itself has an effect on cellular function remains unclear, but *in vitro* studies suggested that lactate has depressive effects on cardiovascular function, and can facilitate cardiac arrhythmias and other adverse effects [31, 32]. In order to study whether K and LA in the SSRBCs have toxic effects on hiPS-CMs, we prepared LA solution, K solution and a mixture of K and LA solution to evaluate their effects on hiPS-CMs, respectively. Our results showed that d0,

d14 and d35 LA had no significant cytotoxicity on CMs. Compared with SSRBCs a similar but weaker cardiotoxicity was observed after treatment at the same concentration of K solution: d0 and d14 K showed no significant effects on NCI and beating pattern, while only d35 K stopped hiPS-CMs beating and showed a moderate decline of NCI value. Since the concentration of K in d35 SSRBCs-treated hiPS-CMs culture medium was much higher than normal human plasma K levels (3.5-5.5mmol/L), it may be the major cause of beating arrest *in vitro* just as shown in the d35 K group. We found that the concentration of LA in d35 SSRBCs-treated hiPS-CMs culture medium was 2.76 mmol/L, and it had no effect on hiPS-CMs growth and beating pattern. Consistent with our results, sodium lactate infusions that raised blood lactate to levels as high as 15 mmol/L did not depress hemodynamic measures in patients after cardiac surgery [33].

Although there are currently no reports on the effects of SSRBCs on hiPS-CMs, some studies have shown that SSRBCs have immunosuppressive effects on immune cells [8]. Muszynski et al. treated 1×10^6 monocytes in 800 μ L of complete medium with 200 μ L of raw SSRBCs, and found that monocyte function was suppressed after exposure to supernatants from d 30 RBCs units compared to monocytes cultured in medium alone. Similar to our hypothesis, this finding implicated that the suppressive effects were induced by soluble mediators in stored RBCs [10]. In addition, they also showed that these immunosuppressive effects of co-incubation with packed RBCs were not related to the concentration of free hemoglobin present. These results may show some support to our findings. Most publications on RBCs storage time are performed in patient groups receiving an average of 1–4 RBCs transfusions [34, 35]. Here, we looked at the observational results of more heavily transfused patient populations simulating a typical blood volume of 2000 ml per 60 kg body weight. The microarray analysis indicated that there was no consistent separation within the naive and d35 K samples, but a large change in gene expression between the d35 SSRBCs and naive group. This was further evidence that d35 K was not the only cause, and there must be other components and related signaling pathways that were involved in the cardiotoxicity.

It is possible that our findings may be questioned based on the observation that clinical patients with K concentrations of 12.0 mmol/L have no cardiac arrest. In fact, increased K associated with RBCs transfusions may be redistributed *in vivo* within minutes. In study of Brown et al, increased hyperkalemia levels caused by rapid blood transfusion declined to below 5.5 mmol/L within 30 minutes. None of the patients with an elevated plasma K required specific treatment for hyperkalemia [36]. In our *in vitro* experiment, the purity of hiPS-CMs was 99% and above, and hence there were not enough cells available for K redistribution.

These data suggest that K in d35 SSRBCs can result in a rapid and complete, but short-term arrest of hiPS-CMs beating, and other metabolic components of RBCs may play a role in the toxicity on hiPS-CMs. Further studies will be required to identify these components and demonstrate their mechanisms.

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Disclosure Statement

The authors declare no competing financial interests.

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