Stored Whole Blood Transfusion Initiate Serum Amyloid A Activation Monitored by Real-Time Dynamic Imaging

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Research

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Abstract

BACKGROUND: Transfusion of stored whole blood (SWB) is increasingly routine practice to resuscitate severe traumatic hemorrhage patients in military operations and civilian emergency centers. It has been well established that transfusion of red blood cells (RBCs) after prolonged storage exerts harmful effects that are mainly mediated by inflammation. Whether the storage lesion that related to inflammation will happen in SWB remains unclear.

METHODS: A hepatocyte SAA (serum amyloid A) specific reporter mouse that facilitated non-invasive imaging of hepatocyte SAA expression was used for the evaluation of acute inflammation and acute-phase reaction after the transfusion of SWB or components separated from end-stage whole blood. Donor C57BL/6 mouse whole blood was used to model an allogeneic transfusion in Balb/C mouse recipients.

RESULTS: End-stage whole blood (14 days of storage) transfusion induced the most significant SAA expression, while 10-day-storage evoked much weaker signal compared to their fresh and 5-day-storage counterparts. It was RBCs rather than white blood cells and plasma-containing platelets that should be responsible for the systemic inflammatory and SAA activation during end-stage whole blood transfusion. Circulatory and hepatic pro-inflammatory cytokines induced the SAA expression in hepatocyte. The macrophage M1 polarization aroused by SWB activated SAA through nuclear transcription factor NF-κB.

CONCLUSION: Storage lesion will also happen during the storage of whole blood, which is related to the change of RBCs after prolonged storage. The side effect induced by systemic inflammation and acute-phase reaction should be taken into consideration before resuscitation by long-term storage whole blood transfusion.

Introduction

Whole blood is increasingly considered to be a better product for resuscitation, which is now being widely used in civilian emergency centers and military operations [1, 2]. It contains all the elements of blood in nearly physiologic ratios, and particularly suitable for the resuscitation of severe traumatic hemorrhage patients[3, 4]. Packed whole blood can be stored for up to 35 days in CPDA-1 under a refrigerated condition[5]. Previous studies suggested that transfusion of older RBCs is associated with acute inflammatory response and enhances the pathogen growth in vitro and in vivo[6, 7]. It is still unclear whether the storage lesion would occur in whole blood components such as RBCs, leukocyte, platelet, and plasma during the storage.

Storage lesions are partly proved as inflammatory reactions during transfusion in vivo[7, 8]. Serum amyloid A (SAA) is one of the positive acute-phase proteins that secreted mainly by hepatocytes. The concentration of SAA in serum can increase up to 1000-fold during an acute inflammatory reaction[9, 10]. It is reported that SAA was able to serve as a more sensitive and reliable biomarker than CRP for the evaluation of the inflammatory reaction level[11]. As a result, in the current study, we employed SAA as a
biomarker for the evaluation of whole blood lesion. ELISA is a common method to detect the content of serum SAA in clinical diagnosis. However, the repeated blood collection will cause certain damage to mouse in the preclinical study, which can also activate the expression of SAA and is not suitable for continuous monitoring of SAA concentrations in the same cohort of mice. Therefore, a SAA-Luc mouse model[10], based on bioluminescence imaging (BLI), was employed to monitor the SAA expression after the transfusion of SWB. Compared with conventional methods, BLI enables both simple in vivo assays and longitudinal studies in the same cohort of mice[12, 13]. The different component of whole blood such as RBC, white blood cell (WBC), platelet-containing plasma (PCP) was also evaluated in our study to further determine the specific components that activate SAA, and the related signal pathway that initiated SAA expression by whole blood transfusion was explored.

**Materials And Methods**

1. **Mice**

BALB/c and C57 mice (male, 6-8 weeks) were bought form Charles river (Beijing) and housed under SPF conditions attach to National Beijing Center for Drug Safety Evaluation and Research.

2. **Mouse whole blood collection and storage**

The whole blood of C57BL/6 mice was collected by cardiac puncture. The CPDA-1 solution with a final concentration as 14% was used for anticoagulation and storage. All the procedures during the blood collection were abode by the principle of sterility strictly. Whole blood was pooled in 50 ml sterile tube (Corning) and stored at 4°C protecting from light. An NWF Leukocyte Reduction Filter (KaiNuo. Biotech Corporation, China) was used for the leucocyte filtration of fresh and stored whole blood.

For the separation of platelet-containing plasma, the whole blood was centrifuged at 200g for 15 minutes, and the supernatant was collected as much as possible which contain the large amount of the platelet. After that, the platelet-containing plasma was centrifuged again at 200g for 15 minutes in a sharp bottom EP sample tube for the removal of residual RBC.

For the separation of RBC, the whole blood was centrifuged at 400g for 15 minutes after the leukocyte depletion. Then, the supernatant was discarded and the layer of residual leukocyte was also thrown away accompanied by partial loss of RBC inevitably. After that, the separated RBC was washed by 10 volume of normal saline 3 times and resuspend with normal saline before transfusion.

3. **Quality control of the stored whole blood**

The detection of hemolysis ratio before transfusion. The blood samples were mix thoroughly by inverting 4-6 times slightly. After that, the hemoglobin concentration and hematocrit were detected by using an automated hematology analyzer (Mindray, BC-2800Vet). The hemoglobin concentration in the supernatant after the centrifugation of mixed SWB represented the free hemoglobin concentrations which
is detected by a free hemoglobin assay kit (Nanjing Jiancheng Bioengineering Institute, A071-1-1). The hemolysis ratio was calculated by the following formula:

\[
\text{Hemolysis ratio} = \frac{(1 - \text{hematocrit}) \times \text{free hemoglobin concentration}}{\text{Total hemoglobin concentration}} \times 100\%
\]

The bacterial contamination in the SWB was evaluated by the inoculated of 500 μl whole blood into LB Solid Medium for 5 days in 37 °C incubator.


The pSAA-Luc plasmid was a kindly gift from Ning Zhang. This plasmid was built by inserting an SAA promoter in front of Luc sequence in a pGL3-Basic vector. Hydrodynamic based gene delivery namely 10% mouse weight volume of normal saline that containing 10 μg naked plasmid was injected through the tail vein rapidly[21]. The bioluminescence imaging was conduct under IVIS Lumina  m (PerkinElmer, Inc.).

5. Elisa

The SAA, IL-1β, IL-6 and TNF-α Elisa kit was bought from R&D systems (MSAA00, MLB00C, M6000B, and MTA00B). The procedure of the Elisa detection was carried out in strict accordance with the operation manual. The OD value in 450nm and 570 nm was read by a MD5 microplate reader.

6. RT-PCR

TRIzol Reagent (Invitrogen, 15596018) was used for the extraction of high-quality total RNA. After that, 1 μg RNA was reverse transcribed into cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO), and amplification of the cDNA was performed for 40 cycles using THUNDERBIRD SYBR qPCR Mix (TOYOBO). 2−ΔΔCt method was used to evaluated the relative expression of the target gene. The primer sequences were listed as Table 1.

Table 1: Sequences of primers for RT-PCR.
### 7. Separation and induction of BMDMs

The femur was separated from BALB/c mice after carbon dioxide euthanasia. Then, the bone marrow was collected by flushing out with a syringe filled with pre-cold 1640 complete medium (10% fetal bovine serum). After that, the cell suspension was obtained by gently dispersed with pipettor and filtration. All of the steps were performed on ice for the improvement of cell viability. The cell suspension was then incubated in 1640 complete medium and 50 ng/mL macrophage colony-stimulating factor (M-CSF, PeproTech, 315-03-100) for 6 d at 37°C in a humidified atmosphere condition filled with 5% CO2. During the incubation and induction, fresh medium with 50 ng/ml M-CSF was added at day 3 and day 5. The BMDMs were ready for use after 6 days of incubation.

### 8. Preparation of Primary Kupffer cells

Mice were anesthetized and livers were perfused with 37 °C preheating in 20 mL D-Hanks (Solarbio, H1045) buffer solution at a rate of 5 ml/min, and then with 37 °C preheating in Hanks (Solarbio, H1025) buffer solution containing 100 U/mL collagenase type IV (Sigma) at a rate of 2 mL/min. The perfusate buffer solution enters the liver through portal vein (PV) and flows out through the inferior vena cava (IVC). The actual duration of 2 ml/min digestion with collagenase type IV was within 6 minutes with periodically applying pressure to the IVC (4-5 seconds each time). The liver would expand and shrink with the repeated pressure to the IVC which could improve the efficiency of digestion. After perfusion of the liver with collagenase type IV, the non-parenchymal cells were separated from hepatocyte by repeated low speed centrifugation, and then the cells were re-suspended by PBS and located on the upper layer of 11.5% and 20% OptiPrep, respectively. After centrifugation (4 °C, 15min, 400g), the upper layer of 20% OptiPrep contained the majority of Kupffer cell and other non-parenchymal cells. We purified Kupffer cell by selective adherence that removed unattached cells thoroughly after 1 h incubation in DMEM complete medium (10% fetal bovine serum) at 37 °C in a humidified atmosphere filled with 5% CO2. Cell viabilities of the primary Kupffer cells were more than 80% and 90%, respectively, in all experiments.
9. Statistical analysis

All of the data were presented as Mean ± SD. The statistical comparisons between different groups were introduced in legend respectively.

Results

1. The dynamic changes of liver SAA after whole blood transfusion at different storage periods.

The whole blood of different storage periods, including 0 day (fresh), 5 days, 10 days and 14 days (end-stage storage), were intravenously transfused with the dose of 300 ul/mouse to reveal the relationships between the storage period and the systemic acute inflammation induction. Donor C57BL/6 mouse whole blood was used to model an allogeneic transfusion in Balb/C mouse recipients. As shown in Figure 1 (A&B), robust signal was observed in 10 days and 14 days storage whole blood treated mice. The signal intensity of the 14 days group was stronger than that of the 10 days group obviously. In terms of 14 days group, the light signals were detectable at 2 h and reached a peak at 8 h post whole blood transfusion and then gradually declined. The dynamic changes of luciferase activity in 10 days group was similar to the 14 days group. The transfusion of 0 days or 5 days storage whole blood did not induce SAA expression within 48 h.

For further confirmation, the dynamic changes of serum SAA concentration and liver SAA and Luc mRNA levels were also measured by ELISA and RT-PCR at 4, 12, and 48 hours post-transfusion, respectively. As shown in Figure 1C, the 14 days whole blood transfusion exert a rapid increase of serum SAA (232.23±28.83 fold compared with the baseline level) at 12 hours. The dynamic changes of liver SAA and Luc mRNA level showed synchronous change (Figure 1 D & E) and were consistent with the result obtained by BIL and ELISA. Taken together, these results suggested that the SWB transfusion induced stronger expression of SAA as the storage time increases, and the 14 days storage whole blood transfusion was able to exert the strongest expression of hepatic SAA. As a result, we chose the fresh and 14 days storage group in the following experiments for further study.

2. WBC in the end stage whole blood did not induce the SAA expression.

WBC may cause transplant rejection because they express a large number of leukocyte antigens and will be eliminated by the recipients[14]. Whether WBC was responsible for the SAA expression induced by allogeneic transfusion of end-stage whole blood need to be further consideration. As a result, normal or leukoreduced whole blood was transfused into the SAA-Luc mice to monitor the SAA expression, respectively. The results showed that end-stage whole blood transfusion was able to stimulate SAA expression regardless of WBC depletion (Figure 2 A&B). These results suggested that allogeneic WBC in the end-stage whole blood did not induce the SAA expression post-transfusion.

3. Storage lesion of red blood cells was responsible for the initiation of SAA expression after the end stage whole blood transfusion.
It is reported that large number of metabolites were generated during the storage of blood cells [15]. So, we evaluated the induction of SAA by PCP from end stage whole blood in the transgenic mice. As shown in Figure 3 (A&B), there is no obvious fluorescence signal at all time points within 48 hours in the PCP group, and the end stage whole blood and fresh whole blood was set as positive and negative control respectively. These results suggested that platelet-containing plasma from end stage whole blood did not induced the SAA expression.

After excluding other component to activate liver SAA expression, we focus on the lesion of red blood cells during storage. As result, we infused separately red blood cells from the end stage whole blood to monitor the activation of SAA expression. The end stage whole blood and fresh whole blood was set as control group as mentioned above. The results showed a similar activation of SAA expression after transfusion of equal amount of whole blood and red blood cell (standardization by red blood cells).

4. Circulatory and hepatic pro-inflammation cytokines initiated the expression of hepatocyte SAA after end stage whole blood transfusion.

It has been reported that SAA was initiated by the transmembrane signal from pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α [16-18]. Next, we quantified these cytokines in serum and liver homogenates by ELISA at 1, 2 and 4 h after end stage whole blood transfusion. As shown in Figure 4 A&B, the concentration of IL-1β, IL-6, and TNF-α in both serum and liver homogenates increase obviously within 4 h in end-stage SWB group. Transfusion of FWB did not induce the secretion of pro-inflammatory cytokines. These results were consistent well with the different intensity of SAA induction observed between FWB and SWB group. To further confirm the initiation of SAA by these pro-inflammatory cytokines after end-stage SWB transfusion, the SAA-Luc mice were adoptively injected by IL-1β, IL-6, or TNF-α cytokines respectively. An obvious signal of SAA activation was detected after cytokines administration as shown in Figure 4 C&D. Taken together, these results suggested that circulatory and hepatic pro-inflammatory cytokines initiated the expression of hepatocyte SAA after end stage whole blood transfusion.

5. The SWB-induced macrophage M1-polarization and pro-inflammatory cytokines secretion contributes to the initiation of SAA expression.

The elimination of senescent RBC was mainly executed by macrophages in liver, spleen and bone marrow [19]. During the phagocytosis of senescent RBC, the macrophage will polarize into classically activated (M1-polarization) and simultaneously release a series of pro-inflammatory cytokines [7, 20]. In this part, we attempted to elucidate whether the production of pro-inflammatory cytokines was coming from activated macrophage. Kupffer cells and bone marrow-derived macrophages (BMDMs) were employed as the cellular model here. As shown in Figure 5A &B, end-stage SWB induced significantly more IL-1β, IL-6 and TNF-α expression than FWB both in Kupffer cells and BMDMs. After that, the polarization of these two cells were evaluated by flow cytometry and RT-PCR. As shown in Figure 5C, the M1 polarization of BMDMs induced by the co-incubation with end-stage SWB was confirmed by the detection of CD86 and CD206 on membrane surface with flow cytometry. The M1 polarization of Kupffer
cells was explored by the detection of M1-related markers including IL-1β, TNF-α and nitric oxide synthase 2 (Figure 5D). Taken together, these results indicated that the pro-inflammation cytokines initiating SAA activation were secreted by M1 polarization macrophages.

6. End-stage SWB induced SAA expression through the NF-κB signal pathway.

The NF-κB-Luc mouse model was used in this part for the detection of NF-κB signal activation as described previously[21]. Figure 6 A&B showed that the luciferase signal in the NF-κB-Luc mouse was detectable at 2-hour post-transfusion and reached a peak at approximately 8-hour, then the signal began to decline. The pretreatment of PDTC (1 mg/kg, NF-κB inhibitor) was able to inhibit NF-κB signal significantly (Figure 6 C&D). To further investigate whether the activation of NF-κB signal pathway was related to SAA activation, we pretreated the SAA-Luc mouse model with PDTC half an hour before the mice were treated with end-stage SWB. As shown in Figure 6 C&E, the SAA activation signal was inhibited by the pretreatment of PDTC obviously. Taken together, these results suggested that end-stage SWB induced SAA expression through the NF-κB signal pathway.

Discussion

In this study, we employed the SAA-Luc mouse to monitor the level of SAA dynamically after the transfusion of whole blood and the corresponding components with different storage time. SAA-Luc mouse has been fully demonstrated in our previous study, which could provide convenience, sensitive, and accurate detection of SAA expression in the liver[10]. We proved that the SAA activation induced by the transfusion of SWB increasing with storage time. After that, we evaluated which components of whole blood activated the SAA expression.

Firstly, we focus our attention on the lymphocytes. The leukoreduction of blood products is the standard of care in many countries to reduce immune consequences of transfusion. However, it is worth noting that leukoreduction of whole blood would cause a significant reduction of whole blood hemostatic function because that the leucocyte filter was also able to decrease the number of platelets[22]. Nathens et al have carried out a single-center, double-blinded randomized control trial comparing transfusion of leukocyte depleted versus non-depleted blood products (n = 268). Their results suggested no significance in 28-day rate of infection (primary endpoint), febrile episodes, organ dysfunction scores, or overall mortality rates[23]. So, the whole blood without leukocyte depletion was evaluated in the current study. Lymphocytes carry plenty of specific human leucocyte antigens which might be recognized by the immune system of blood recipient and caused host inflammatory and immune cascade reaction[24].

Secondly, it is reported that the transfusion-related GVHD was caused by viable or immunocompetent lymphocytes that from the transfusion of stored blood products[25, 26]. Moreover, leukocyte may release some pro-inflammatory factors during storage[27]. Therefore, it is reasonable to speculate that the SAA activation after end-stage SWB transfusion might cause by the inflammatory and immune response related to leukocyte. As a result, the donor C57BL/6 mouse whole blood without leukocyte depletion was used to model an allogeneic transfusion in Balb/C mouse recipients as described previously[7]. Our
results suggested that whether leukocyte was depleted or not does not influence the SAA activation after end-stage SWB transfusion and the leukoreduction of whole blood for transfusion in trauma patients should be reconsidered.

Several studies reported that the metabolites are altered during RBC storage[15, 28]. There are more complicated cell compositions in whole blood. So, the metabolites alteration of SWB should take into consideration in the induction of hepatocyte SAA. And other studies indicated that platelets represent the main source of inflammatory and/or allergic hazards in transfusion; this is linked with processing and storage lesions but also with the platelet physiology itself[29]. However, it is reported that platelet-rich plasma injection was effective in anti-inflammatory treatment in rheumatoid arthritis patients[30], and it promotes skeletal muscle healing in association with decreasing inflammation and apoptosis of injured skeletal muscle[31]. As a result, platelet and the accumulation of metabolites in plasma may also contribute to the activation of liver SAA. However, no obvious signal was monitored after PCP infusion. The storage lesion of platelets and metabolic change in plasma will not induced the inflammatory response and SAA activation. These results convince us that the storage lesions of RBC in end-stage whole blood appear to be the only component that activates SAA expression.

The storage lesion of RBCs has been described clearly before[6, 7, 32]. The transfusion of end-stage RBCs was able to promote inflammation and bacteria proliferation[7]. The side effects of end-stage RBCs transfusion were due to the monocyte-macrophage system that serves as the first line of defense of the body against foreign substances[33]. The liver and spleen were the main organs that contain the majority of macrophages in the body and responsible for the elimination of end-stage RBCs[7, 34]. In the current study, Kupffer cells and BMDMs served as the cellular model for the phagocytosis and inflammation study, which represent the local macrophage and transient macrophage respectively. The selection of cell models was on the base of the previous study that both of the local macrophages (Kupffer cells) and the transient macrophage (on-demand macrophage) contributed to the removal of the senescent or damaged RBCs and cytokines secretion[34]. We have ruled out the possibility that whole blood components except for RBCs induced the inflammatory reaction. The store lesion of whole blood mainly comes from the change of RBCs after long time storage and our results consistent well with the previous studies mentioned above that the transfusion of end-stage whole blood induced the M1-polarization of macrophages and the inflammatory reaction[7, 34]. The pro-inflammatory cytokines from M1-polarized macrophages initiated the expression of SAA through NF-κB signal pathway which was also proved by the others' work [13, 17].

Conclusion

The SAA-Luc mouse was employed for evaluating the storage lesion of whole blood in the current study. Our results suggested that storage lesion related to inflammation will also happen during the storage of WB and the enhancing inflammatory response after transfusion was correlated with the increased storage time of WB. After screening different components in WB, we found that RBCs should be responsible for the systemic inflammatory and SAA activation during end-stage WB transfusion, and the
M1 polarized macrophage was the main source of such inflammatory cytokines. As a result, according to
the current study, we recommended that the side effect induced by systemic inflammation and acute-
phase reaction should be taken into consideration before resuscitation by long-term storage whole blood
transfusion.

**Declarations**

**Ethics approval and consent to participate**

All the animal experiments involved in this study were approved by the Animal Ethics Committee of
National Beijing Center for Drug Safety Evaluation and Research.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets generated and/or analyzed during the current study are available from
the corresponding author by reasonable request.

**Competing interests**

The authors have no conflicts of interest to disclose.

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**Authors’ contributions**

ZLS, WXH, ZYL, WZJ and LL responsible for design of this work. ZYL WZJ LL ZQQ, SSJ, MP and LLP
performed the related experimental operations. Data analysis and article writing was completed by ZYL
WZJ and LL. All authors approved the final manuscript.

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**References**


Figures
Figure 1

The dynamic changes of hepatic SAA after transfusion of whole blood at different storage periods. (A) Bioluminescence imaging of SAA expression by SAA-Luc mouse after transfusion of whole blood with 0, 5, 10, 14 days of storage; (B) The quantitative analysis of signal intensity in (A); (C) Serum SAA concentration measured by Elisa after transfusion of end-stage storage whole blood; (D & E) Liver SAA and Luc level measured by RT-PCR after transfusion of end-stage storage whole blood. n=4, * represented the comparison with control group. *p < 0.05, One-way ANOVA followed with Tukey multiple comparison was employed for the statistical analysis.
Figure 2

WBC in the end stage whole blood did not induced the SAA expression. (A) Bioluminescence imaging of SAA expression by SAA-Luc mouse after transfusion of whole blood with or without leukocyte-filtered; (B) The quantitative analysis of signal intensity in (A).

Figure 3

Storage lesion of red blood cells was responsible for the initiation of SAA expression. (A) Bioluminescence imaging of SAA expression by SAA-Luc mouse after transfusion of whole blood, PCP, and RBC; (B) The quantitative analysis of signal intensity in (A).
Figure 4

Circulatory and hepatic pro-inflammation cytokines initiated the expression of hepatocyte SAA after end stage whole blood transfusion. (A) Serum IL-1β, IL-6 and TNF-α concentration after measured by Elisa after the transfusion of end-stage whole blood transfusion. (B) IL-1β, IL-6 and TNF-α concentration in liver homogenate measured by Elisa after the transfusion of end-stage whole blood transfusion; (C) Bioluminescence imaging of SAA expression by SAA-Luc mouse after i.p. injection of IL-1β, IL-6, and TNF-α at a dose of 1 μg per mouse, respectively. (D) The quantitative analysis of signal intensity in (C). n=4, * represented the comparison with control group. *p < 0.05, One-way ANOVA followed with Tukey multiple comparison was employed for the statistical analysis.
Figure 5

The SWB-induced pro-inflammatory cytokines secretion and macrophage M1-polarization. (A) IL-1β, IL-6 and TNF-α secretion by BMDMs after 8 hours of co-incubation with FWB and SWB; (B) IL-1β, IL-6 and TNF-α secretion by Kupffer cells after 8 hours of co-incubation with FWB and SWB; (C) The abundance of CD86 and CD206 on BMDMs after 8 hours of co-incubation with FWB and SWB; (D) The M1 and M2 polarization related gene expression in Kupffer cells after 8 hours of co-incubation with FWB and SWB. n=4, * represented the comparison with control group. *p < 0.05, One-way ANOVA followed with Tukey multiple comparison was employed for the statistical analysis.
Figure 6

End-stage SWB induced SAA expression through the NF-κB signal pathway. (A) Bioluminescence imaging of NF-κB activation by NF-κB -Luc mouse after transfusion of FWB and SWB; (B) The quantitative analysis of signal intensity in (A); (C) The modulation of NF-κB activation and SAA expression by the pretreatment of PDTC half an hour before the transfusion of SWB, which is monitored by the bioluminescence imaging; (D&E) The quantitative analysis of luciferase signal in (C) n=4, * represented the comparison with control group. *p < 0.05, One-way ANOVA followed with Tukey multiple comparison was employed for the statistical analysis.