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Transfusion of old red blood cells induces neuroinflammation and cognitive impairment

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Abstract

Objectives—Transfusing red blood cells (RBC) stored for longer than 14 days (old RBC) in humans is common. This transfusion can injure organs, such as lungs and kidneys. We determined whether transfusion with old RBC injured brain.

Design—Prospective, controlled, animal study

Setting—University research laboratory

Subjects—Adult male Sprague–Dawley rats

Interventions—Six-month old Sprague–Dawley rats lost 20% total blood volume and then received RBC prepared from equal volume of the lost blood. RBC was stored for 1 day (fresh RBC) or 7 days (old RBC, storage lesions similar to those of human RBC stored for 28 days). Some rats received intravenous cell-free hemoglobin. These rats were not subjected to hemorrhage and RBC transfusion.

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Measurements and Main Results—Rats were subjected to Barnes maze and fear conditioning tests from one week after blood transfusion. Rats transfused with old RBC but not fresh RBC took a longer time to identify the target hole in the Barnes maze and had less context-related fear conditioning behavior than control rats. Old RBC significantly increased interleukin 6 and ionized calcium-binding adapter molecule 1 in the hippocampus at 24 h after the transfusion. These effects were attenuated by sulforaphane and minocycline, an antibiotic with anti-inflammatory property. Old RBC solution had a higher concentration of cell-free hemoglobin. Sulforaphane increased haptoglobin, a chelator of cell-free hemoglobin. Rats received cell-free hemoglobin had a pattern of neuroinflammation and impairment of learning and memory similar to that of rats received old RBC.

Conclusions—These results provide initial evidence to suggest that transfusion of old RBC induces neuroinflammation and impairment of learning and memory. These effects may be mediated by cell-free hemoglobin.

Keywords

learning and memory; neuroinflammation; old red blood cell; transfusion

Introduction

Blood transfusion has been practiced since 1930s (1). It can increase oxygen-carrying capacity of the blood, improve hemostasis and provide volume expansion (1, 2). In some cases, blood transfusion is a life-saving measure. However, blood transfusion has significant side effects, such as the risks of transmitting infectious diseases and causing immunological reactions (1).

The Food and Drug Administration allows transfusion of red blood cells (RBC) that have less than 1% erythrocytes lysed in the storage bag and 25% stored cells cleared from the recipient within 24 h after the transfusion (3). This requirement permits transfusion of RBC stored *ex vivo* in the preserving solution by current method for up to 42 days. The average storage time of transfused RBC in 2007 was ~20 days in the U.S.A. (4), suggesting that transfusion of blood stored for > 14 days (old RBC) is common in clinical practice. However, transfusion with RBC older than 14 days has been shown to increase pulmonary complications, kidney failure, multi-organ failure, death and the length of hospital stay in patients in many studies (5, 6), although no increase of these poor outcome has been reported in few studies (6, 7). Interestingly, the effects of transfusion with old RBC on brain functions and structures have not been described.

The mechanisms for old RBC-induced organ injury are not fully understood. Recent studies suggest an important role of cell-free hemoglobin in this effect (8, 9). Cell-free hemoglobin can induce inflammation (10), a pathological process involved in almost all acquired diseases (11). Thus, we hypothesize that transfusion with old RBC can induce cognitive impairment and neuroinflammation and that these effects are mediated by cell-free hemoglobin. To test these hypotheses, we transfused old and fresh RBC to rats and tested their cognitive functions. Minocycline and sulforaphane were used in the study. Minocycline

is an antibiotic with anti-inflammatory property (12). Sulforaphane can increase the expression of haptoglobin (13) that can chelate cell-free hemoglobin.

Materials and Methods

The animal protocol was approved by the institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA, USA). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80–23) revised in 2011.

Animal groups

Six-month old male Sprague–Dawley rats (500 - 600 g) from Charles River Laboratories Inc. (Wilmington, MA) were randomly assigned to one of the six groups: (1) control group in which animals underwent anesthesia, vascular cannulation and monitoring for 90 min, but were neither hemorrhaged nor resuscitated; (2) minocycline group in which rats received minocycline and other manipulations for control animals; (3) fresh RBC group in which rats received fresh RBC; (4) old RBC group in which rats received old RBC; (5) old RBC and minocycline group in which rats received old RBC and minocycline; and (6) old RBC and sulforaphane group in which rats received old RBC and sulforaphane. These animals were used for brain pathological assessment (one cohort of rats, $n = 6$ per group) and learning and memory testing (another cohort of rat, $n = 9$ per group).

The third cohort of rats was randomized to three groups ($n = 8$ per group): (1) control group; (2) fresh RBC group; and (3) old RBC group. They received identical procedures as described above for each group. These rats were used to examine the effects of hemorrhage and RBC transfusion on cerebral blood flow and general blood chemical contents.

Additional rats in three groups was studied: (1) control group; (2) hemoglobin group in which rats received cell-free hemoglobin; and (3) hemoglobin and sulforaphane group in which rats received cell-free hemoglobin and sulforaphane. These animals were used for brain pathological assessment (one cohort of rats, $n = 6$ per group) and learning and memory testing (another cohort of rat, $n = 9$ per group).

Since there was no prior study on the effects of transfusion of old RBC on the brain in the literature, we did not have good data for use to calculate sample size. Thus, we arbitrarily decided the sample sizes of each experiment based on our previous studies on surgery-induced neuroinflammation and impairment of learning and memory (14–16).

Anesthesia

As we described before with some modifications (17), anesthesia was induced by placing rats in a chamber gassed with 3% isoflurane in oxygen. The rats then were intubated with a 14-gauge catheter and mechanically ventilated. Anesthesia was maintained by isoflurane carried by 100% O₂ to maintain end tidal isoflurane concentration at 2.0%. The inhaled and exhaled gas concentrations were monitored continuously with a Datex™ infrared analyzer (Capnomac, Helsinki, Finland). The ventilator settings were as follows: 2 ml as the tidal volume and respiratory rate at 60 breaths/min. The settings were adjusted to maintain the

end tidal CO₂ at ~32 mm Hg. Rectal temperature was maintained at 37°C with the aid of servo-controlled warming blanket (TCAT-2LV, Physitemp instruments Inc., Clifton, NJ). Heart rate and SpO₂ were measured continuously during anesthesia with a MouseOx™ Pulse Oximeter (Harvard Apparatus, Holliston, MA). The total duration of anesthesia lasted for 1.5 h. Rats were extubated when righting reflex was recovered.

Minocycline and sulforaphane application

Minocycline (Catalog No. M 9511, Sigma-Aldrich, St. Louis, MO) at 40 mg/kg in normal saline (NS) was administered intraperitoneally after tracheal intubation. Sulforaphane (Catalog No. S8044, LKT Laboratories, Inc. St. Paul, MN) at 5 mg/kg (dissolved in corn oil first and then diluted in NS to make the final solution of 10% corn oil) was administered intraperitoneally 24 h before anesthesia and immediately after tracheal intubation. The dosage was chosen based on previous studies (12, 18-20). Same volume of NS was given intraperitoneally to other groups that did not receive minocycline and sulforaphane.

Rat RBC preparation

RBCs were prepared from male Sprague–Dawley rats using sterile technique, as described before with some modifications (21). Briefly, whole blood was withdrawn into a sterile polypropylene tubes containing citrate phosphate dextrose adenine (CPDA)-1 (CPDA-1: blood at 1:7), and centrifuged at 3600 g for 5 min at 4°C. The platelets, plasma and leukocytes were removed. The RBCs were stored at 4°C until the experiments. The prepared RBC solution had a hematocrit of $70 \pm 5\%$. RBC used for transfusion as fresh cells were administered within 1 day of collection. Old RBC was those stored for 7 days. We treated these cells as old RBC because a previous study has shown that rat RBCs stored for 7 days in CPDA-1 show storage lesions similar to that of human RBCs stored for 28 days in terms of the depletion of ATP and 2,3-diphosphoglycerate and the loss of cell deformability (22). These prepared RBCs were transfused to rats of a different cohort after hemorrhage. This practice is common (23, 24). In addition, mixing blood from different rats very rarely induces cell aggregation (25). To determine whether stored RBC solution was contaminated by bacteria, 8 randomly selected RBC solutions that had been stored for 7 days were inoculated to agar plates (0.2 ml RBC per plate). To create a positive control, some plates were touched by a finger of the experimenter. The plates were kept in an incubator at 37°C for 7 days after the inoculation.

Transfusion protocol

After tracheal intubation, the tail artery and right or left lateral tail vein were cannulated with a 24G intravenous catheter. The arterial line was used to measure blood pressure (Columbus Instrument International Corp., Columbus, OH) and for withdrawing blood from the animals (controlled hemorrhage). The intravenous line was used to infuse blood and fluids. We aimed to withdraw blood of 20% total blood volume (BV). The total BV was estimated according to the following equation: total BV (ml) = $0.77 + 0.06 \times \text{body weight (g)}$ (26). After baseline measurements for 20 min, rats received intravenous Ringer's lactate solution at the planned shed blood volume at a constant rate over 10 min. Then 20% of the total BV was slowly withdrawn from the tail artery over 10 min. The stored RBCs isolated from the same shed volume of total blood were warmed to room temperature and then mixed

with NS at the shed blood volume and Ringer's lactate solution at the shed blood volume. This mixture was transfused into the rats over 20 min. This resuscitation regime was designed to mimic clinical practice at 3:1 crystalloid:RBC ratio (27). At the completion of RBC transfusion, arterial blood was drawn for assessing the contents of blood gases and electrolytes using an analyzer (IRMA TruPoint Blood Analysis System, International Technidyne Corporation, Edison, NJ) and the CC cartridge (Reference No.: 039903, International Technidyne Corporation). Blood lactate concentrations were determined in the Clinical Pathological Laboratory of University of Virginia Hospital (Charlottesville, VA).

Cell-free hemoglobin application

Human hemoglobin (Catalog No.: H7379, Sigma-Aldrich, St. Louis, MO) at 200 mg in 10 mL NS was infused intravenously for 40 min after anesthesia intubation. The dosage was chosen based on a previous study (28). The same volume of NS was given to control rats. We used human hemoglobin because we could not find a source to purchase rat hemoglobin. In addition, rat hemoglobin is similar to human hemoglobin (29). Finally, results from human hemoglobin may be clinically relevant.

Cerebral blood flow monitoring

Animals were anesthetized by isoflurane. Two holes at 0.8 mm posterior to bregma and 2.5 mm from midline, one on each side, were made through the cranium. The tip of the probe was directly against the brain tissues. The probe was connected to a blood flow monitor (Moor Instruments DRT4, Moor Instruments Limited, Millwey, England). Blood flow at each side of the brain was recorded at the following time points: time 0 corresponded to the end of baseline measurement; time 10 was the end of Ringer's lactate solution transfusion; time 20 corresponded to the end of hemorrhage; time 40 was the end of transfusion of RBC and fluids. The data at the other time points was normalized by the corresponding data at time 0.

Barnes maze

One week after transfusion with RBC or cell-free hemoglobin, rats were subjected to Barnes maze as we described before (30) to test their spatial learning and memory. Barnes maze is a circular platform with 20 equally spaced holes (SD Instruments, San Diego, CA). One hole was connected to a dark chamber that was called target box. Each time, animals were placed in the middle of the platform and encouraged to find the target box by aversive noise (85 dB) and bright light (200 W) shed on the platform. They went through a spatial acquisition phase that consisted of training on 4 days with 2 trials per day, 3 min per trial and 15 min between each trial. The reference memory of the rats was tested on day 5 and day 12. One trial on each of these two days was performed. The rats were not subjected to any tests during the period from day 5 to day 12. The latency to find the target box during each trial was recorded with the assistance of ANY-Maze video tracking system (SD Instruments).

Fear conditioning

One day after Barnes maze test, rats were subjected to fear conditioning test as we described before (30). Each rat was placed in a test chamber wiped with 70% alcohol and subjected to

3 tone-foot shock pairings (tone: 2000 Hz, 85 db, 30 s; foot shock: 1 mA, 2 s) with an intertrial interval 1 min in the chamber placed in a relatively dark room (training sessions). The rat returned to home cage 30 s after the conditioning training and was placed back to the chamber 24 h later for 8 min in the absence of tone and shock. The amount of time with freezing behavior was recorded in an 8 s interval. The animal was placed 2 h later in a test chamber that was different in context and smell from the first test chamber and was in a relatively light room. Freezing behavior was recorded for 3 min without the auditory conditioning stimulus. The auditory stimulus then was turned on for 3 cycles, each cycle for 30 s followed by 1-min inter-cycle interval (4.5 min in total). The freezing behavior in this 4.5 min was recorded. Freezing behavior recorded in the video was evaluated by an observer who was blind to group assignment.

Brain tissue harvest

Rats were deeply anesthetized with isoflurane and perfused transcardially with NS at 24 h after the RBC transfusion or cell-free hemoglobin infusion. The right hippocampus and cerebral cortex were dissected out immediately for enzyme-linked immunosorbent assay (ELISA) of interleukin (IL)-1 β and IL-6. The left cerebral hemisphere at Bregma -3 to -6 was harvested for Immunofluorescence staining.

Quantification of IL-1 β and IL-6

The brain levels of IL-1 β and IL-6 were determined with Quantikine ELISA kits (Catalogue No.: RLB00 and R6000B, respectively, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions and as we described before (30). Briefly, brain tissues were homogenized on ice in 20 mM Tris-HCl buffer (pH 7.3) containing protease inhibitors cocktail (Catalogue No.: P2714; Sigma, St Louis, MO). Homogenates were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was then ultra-centrifuged at 150,000 g for 2 h at 4°C. Bradford protein assay of the supernatant was performed for each sample. The supernatant was used in ELISA. The quantity of cytokines in each brain sample was normalized by its protein contents.

Immunofluorescence staining

The tissue preparation of immunofluorescent staining of brain sections were performed as we described previously (31). Brain slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline at 4°C for 24 h and embedded in paraffin. Five-micrometer thick paraffin coronal sections were cut sequentially and mounted onto slides. The sections were heated at 60°C for 1.5 h and deparaffinized using xylene and graded ethanol. Antigen retrieval was performed by boiling sections in a Tris base/ethylenediaminetetraacetic acid (Tris/EDTA) buffer (pH = 9.0) containing 0.05% tween-20 for 20 min. Sections were washed in Tris-buffered saline (TBS) plus 0.025% triton-X 100. They were then blocked in 10% donkey serum with 1% bovine serum albumin in TBS for 2 h at room temperature. The sections were incubated with rabbit polyclonal anti-ionized calcium-binding adapter molecule 1 (Iba-1) antibody (1:500, catalogue number: 019-19741; Wako Chemicals USA, Richmond, VA) overnight at 4°C. Sections were rinsed in TBS with 0.025% triton-X 100, followed by incubation for 1 h with donkey anti-rabbit IgG Alexa Fluor 488-conjugated secondary antibody (1:200 dilution, catalogue number: A21206, Life Technologies, Grand

Island, NY). After washing, the sections were counterstained with Hoechst 33342 (1:1000, catalogue number: 062249, Thermo Scientific, Pittsburgh, PA) for 5 min, then rinsed, and mounted with the aid of Vectashield mounting medium. A negative control omitting the incubation with the primary antibody was performed.

As we described before (14), image quantification was performed in 3 non-overlapping fields randomly acquired in the hippocampal sub-regions or cortex at $\times 100$ magnification on an Olympus DP70 microscope. Three sections per rat were imaged. The number of pixels per image with intensity above a predetermined threshold level was quantified using ImageJ 1.47n software (National Institutes of Health, Bethesda, MD). The immunoreactivity was reflected by the percentage pixels with intensity above a threshold level in the total pixels of the imaged field. All quantitative analyses were performed in a blind manner.

Plasma haptoglobin quantification

Immediately after tail artery cannulation, 0.5 ml blood of control rats or rats received sulforaphane was collected into EDTA tubes and centrifuged at 3600 g for 5 min at 4°C. The plasma was collected and stored at -80°C until use. The level of haptoglobin in the plasma was measured using solid-phase ELISA kits (Immunology Consultants Laboratory Inc., Portland, OR) and a Biorad Model 680 microplate reader (Biorad, Hercules, CA) according to the manufacturer's instructions. All assays were carried out in duplicates.

Quantification of cell-free hemoglobin

Same volume of NS (3 ml NS to 3 ml RBC) was added to control (stored for 1 h), fresh RBC (stored for 1 day) or old RBC (stored for 7 days) and mixed gently. The sample was centrifuged at 3600 g for 5 min at 4°C. The supernatant was stored at -80°C . Cell-free hemoglobin concentration in the supernatant was determined with a QuantiChrom Hemoglobin Assay Kit (BioAssay Systems, Hayward, CA).

Statistical analysis

Results are presented as means $\pm$ S.E.M. ($n = 6$). The data from the training sessions of Barnes maze test within the same group were tested by one-way repeated measures analysis of variance followed by Tukey's post-hoc pairwise comparison testing. The between group data from the training sessions of Barnes maze were analyzed by two-way repeated measures analysis of variance followed by Tukey's post-hoc pairwise comparison testing. The blood pressure, body temperature and cerebral blood flow within the same group were analyzed by one-way repeated measures analysis of variance followed by Tukey's post-hoc pairwise testing. All other data, with more than two groups, were analyzed by one-way analysis of variance followed by the Tukey's post-hoc pairwise comparison testing if the data were normally distributed, or one-way analysis of variance on the ranks followed by the Tukey's post-hoc pairwise comparison testing if the data were not normally distributed. The Student's t test or the Mann-Whitney Rank Sum test was used to analyze non-repeated measure data from two groups. A $p = 0.05$ decision rule was utilized as the null hypothesis rejection criterion for all statistical comparisons. All statistical analyses were performed with the SigmaStat (Systat Software, Inc., Point Richmond, CA, USA).

Results

No animals died during the experiments and the following observation period. All animals contributed data for analysis. No bacterial colonies were grown up in the agar plates inoculated with the 8 blood samples stored for 7 days at 24 h or 7 days after the inoculation. However, numerous bacterial colonies were identified if a bare finger touched the agar plates (data not shown).

RBC transfusion reversed blood loss-induced hypotension and did not affect cerebral blood flow

Removing blood at 20% total BV decreased the systolic arterial blood pressure. This decrease reached significance when compared with the control animals or their initial blood pressures before fluid transfusion and blood withdraw. However, this decrease was reversed by fluid and RBC transfusion. At the end of experiments, the systolic arterial blood pressure was not different among the groups and from their initial blood pressures (Fig. 1). These blood pressure changes were reproduced in another cohort of rats. However, blood removal and RBC transfusion did not change the body temperature and cerebral blood flow (Fig. 2).

RBC transfusion did not change general blood chemical concentrations in rats

RBC solution stored for 7 days had no change in sodium concentration but had higher concentrations of potassium and lactate than RBC solution stored for 24 h (Fig. 3). However, transfusion of RBC stored for 7 days did not affect the concentrations of blood electrolytes, lactate and arterial blood gases of the recipients. Rats that received transfusion of RBC stored for 1 day or 7 days had the same total blood hemoglobin levels as those of control rats at the end of RBC transfusion (Figs. 3 and 4).

Transfusion of old RBC induced learning and memory impairment

Rats in all groups except for the rats received transfusion of old RBC required less time on training day 4 than on training day 1 to find the target hole (Fig. 5A). Transfusion of old RBC had a significant effects on the time to find the target hole in the training sessions [$F(1,16) = 10.207$, $P = 0.005$]. Transfusion of fresh RBC was not a significant factor to affect the rats to find the target hole in the training sessions [$F(1,16) = 2.857$, $P = 0.109$]. Rats transfused with old RBC also took a longer time than control animals to find the target hole at 1 day and 8 days after the training sessions. This effect was attenuated by sulforaphane at 1 day after the training sessions. Sulforaphane tended to attenuate the effects of old RBC transfusion on the time to identify the target hole at 8 days after the training sessions. However, this effect did not reach statistical significance ($P = 0.064$). Similar situation occurred to minocycline at 1 day after training sessions ($P = 0.230$, old RBC transfusion group vs. old RBC transfusion plus minocycline group) (Figs. 5B and 5C).

Rats transfused with old RBC had decreased context-related freezing behavior in the fear conditioning tests compared with control rats. The context-related freezing behavior in the other groups was not different from that of control group (Fig. 6A). There was no difference in the tone-related freezing behavior among all groups of rats (Fig. 6B).

Possible role of cell-free hemoglobin and neuroinflammation in old RBC-induced learning and memory impairment

Fresh RBC solution did not have an increased concentration of cell-free hemoglobin [5.9 ± 1.0 mg/ml (blood stored for 1 day) vs. 5.2 ± 0.2 mg/ml of control (blood stored for 1 h), $n = 6 - 7$, $P = 0.571$]. However, cell-free hemoglobin was significantly increased in the old RBC solution [17.7 ± 1.0 mg/ml (blood stored for 7 days), $n = 6$, $P < 0.001$ compared with control]. As expected, sulforaphane increased haptoglobin concentrations in the blood of rats (3.37 ± 0.13 mg/ml vs. 1.36 ± 0.36 mg/ml of control, $n = 7 - 9$, $P < 0.001$).

IL-6 in the hippocampus was significantly increased at 24 h after the transfusion with old RBC. This increase was attenuated by sulforaphane and minocycline. There were no significant changes in the amount of IL-6 in the cerebral cortex and IL-1 β in the cerebral cortex and hippocampus at this time point (Fig. 7). Iba-1 expression was increased in the cerebral cortex and hippocampus at 24 h after the old RBC transfusion. This increase was attenuated by sulforaphane and minocycline (Fig. 8). Transfusion with fresh RBC did not affect the amount of IL-6, IL-1 β and Iba-1 (Figs. 7 and 8).

Cell-free hemoglobin induced learning and memory impairment and neuroinflammation

Transfusion of cell-free hemoglobin to animals without blood loss increased systolic arterial blood pressure (Fig. 1B), possibly due to scavenger of nitric oxide. This transfusion had a significant effects on the time to find the target hole in the training sessions of Barnes maze testing [$F(1,16) = 16.553$, $P < 0.001$]. Rats that received cell-free hemoglobin also took a longer time than control animals to find the target hole at 1 day and 8 days after the training sessions. These effects were attenuated by sulforaphane (Figs. 9A and 9B). Rats that received cell-free hemoglobin also tended to have less context-related freezing behavior (overall $P = 0.332$ for one-way analysis of variance for the analysis of the data from the three groups of rats) in the fear conditioning. The tone-related freezing behavior was not affected (Fig. 9C). This pattern of learning and memory impairment is similar to that of the rats that received old RBC. In addition, cell-free hemoglobin infusion increased Iba-1 immunostaining in the cerebral cortex and hippocampus and IL-6 concentration in the hippocampus but did not affect IL-6 in the cerebral cortex and IL-1 β in the hippocampus and cerebral cortex at 24 h after the infusion (Fig. 10). This pattern of changes is also similar to that caused by transfusion of old RBC. Of note, the increase of Iba-1 in the cerebral cortex and hippocampus and IL-6 in the hippocampus was attenuated by sulforaphane (Fig. 10).

Discussion

Transfusion with RBC older than 14 days is associated with increased pulmonary complications and kidney failure in human (5, 6). However, old RBC transfusion-induced brain injury has not been reported in humans or animals. Our study showed that transfusion with old RBC but not with fresh RBC impaired the performance of rats in Barnes maze and fear conditioning testing. Transfusion of old RBC also increased IL-6 and Iba-1 in the brain. These results clearly indicate that transfusion with old RBC induced cognitive dysfunction and neuroinflammation. Since context-related fear conditioning was affected after old RBC

transfusion, this transfusion appears to impair hippocampus-dependent learning and memory in these rats (30).

Blood cell storage lesions are now well-documented (6). *Ex vivo* stored cells have decreased 2, 3-diphosphoglycerate and ATP. They have increased biologically active lipids and microvesicles. These biochemical changes increase the rigidity of the cells for them to be hemolyzed, decrease oxygen-carrying capacity, alter cell adhesiveness and aggregability, and promote inflammation (6, 32). Our results showed that RBC solution stored for 7 days had an increased concentration of cell-free hemoglobin, potassium and lactate in the solution, suggesting significant storage lesions in these old RBCs.

Cell-free hemoglobin can induce inflammation (10). Hemoglobin and its metabolite heme can induce expression of various adhesion molecules and increase vascular permeation (33). Cell free-hemoglobin can avidly bind nitric oxide. Nitric oxide has been shown to inhibit cytokine-induced adhesion molecule expression (34). The cell-free hemoglobin may play an important role in the development of neuroinflammation after transfusion with old RBC in our study because transfusion with fresh RBC solution that did not contain increased cell-free hemoglobin did not induce neuroinflammation. Transfusion with old RBC solution that contained a large amount of cell-free hemoglobin induced neuroinflammation. Sulforaphane induced the expression of haptoglobin, a hemoglobin binding protein (13), also attenuated old RBC transfusion-induced neuroinflammation. More importantly, intravenous infusion of cell-free hemoglobin increased IL-6 in the hippocampus and Iba-1 in the cerebral cortex and hippocampus, suggesting that cell-free hemoglobin induces neuroinflammation.

Neuroinflammation may contribute to the learning and memory impairment after transfusion with old RBC. Sulforaphane inhibited neuroinflammation and also partially attenuated the learning and memory impairment after the transfusion. Minocycline, an antibiotic with anti-inflammatory property (12), reduced neuroinflammation and trended to attenuate learning and memory impairment after transfusion with old RBC. In addition, we and others have shown that neuroinflammation may be the underlying pathology for postoperative cognitive dysfunction (12, 14), a clinical syndrome characterized by learning and memory impairment after surgery (35-37). Minocycline has been shown to reduce surgery- or anesthetic-induced neuroinflammation and impairment of learning and memory in previous studies (12, 38, 39). Also, cell-free hemoglobin induced neuroinflammation and impairment of learning and memory. Sulforaphane attenuated these effects. Thus, our results suggest the following pathological sequences for transfusion with old RBC to induce learning and memory impairment: cell-free hemoglobin-neuroinflammation-cognitive impairment.

Our findings may have significant implications. Transfusion of RBC is a common practice, especially during the perioperative period. About 50% patients in the intensive care units and ~50% patients during the peri-cardiac surgery receive blood transfusion (1, 6, 40). A significant amount of patients after non-cardiac surgery also have blood transfusion. For example, ~20% patients receive blood after total joint arthroplasty (41), a procedure commonly performed in elderly patients. In addition, transfusion of old RBC (> 14 days) frequently occurs in clinical practice (4). Thus, transfusion with old RBC during perioperative period may contribute to the development of postoperative cognitive

dysfunction. This transfusion may also induce subtle and yet noticeable cognitive impairment in patients without surgery.

To simulate clinical situation, we subjected rats to a blood loss of 20% total BV before RBC transfusion. This blood loss led to significant hypotension. However, the blood loss and hypotension may not be the cause of neuroinflammation and learning and memory impairment because rats transfused with fresh RBC had a similar degree of blood loss and hypotension but did not have neuroinflammation and learning and memory impairment. Also, the hypotension and RBC transfusion did not affect cerebral cortical blood flow and body temperature. The transfusion did not affect the concentrations of blood gases, electrolytes and lactate in the recipient rats. Finally, RBC solution stored for 7 days was not contaminated by bacteria. These results suggest that the neuroinflammation and impairment of learning and memory after transfusion of old RBC are not due to hemodynamic alterations and general blood chemical changes.

We used sulforaphane to increase haptoglobin to imply the role of cell-free hemoglobin in the neuroinflammation and learning and memory impairment. Obviously, sulforaphane has multiple pharmacological effects in addition to increasing haptoglobin expression. For example, sulforaphane can inhibit histone deacetylase activity (42). Sulforaphane reduces amyloid beta-induced cytotoxicity and improves cognitive functions after brain trauma possibly through its anti-oxidative property (20, 43). These effects may contribute to sulforaphane-induced reduction of neuroinflammation and impairment of learning and memory after old RBC transfusion. Thus, giving haptoglobin intravenously would have provided better evidence to indicate the involvement of cell-free hemoglobin in the neuroinflammation and learning and memory impairment after old RBC transfusion. However, it is difficult to use haptoglobin under the *in vivo* condition because of the prohibited cost of the protein. Nevertheless, cell-free hemoglobin induced a pattern of neuroinflammation and impairment of learning and memory similar to that caused by old RBC transfusion. Together, our results strongly suggest the role of cell-free hemoglobin in the learning and memory impairment caused by old RBC transfusion.

Conclusions

We have shown that transfusion with old RBC but not fresh RBC induces neuroinflammation and learning and memory impairment. These pathological changes may be caused by cell-free hemoglobin. Chelating cell-free hemoglobin or inhibiting neuroinflammation may be effective methods for attenuating learning and memory impairment after transfusion of old RBC.

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Fig. 1. Systolic arterial blood pressure changes in various groups

Six-month old male Sprague–Dawley rats lost 20% total blood volume and then received red blood cells (RBC) prepared from equal volume of the lost blood. Another cohort of rats received intravenous 200 mg cell-free hemoglobin. A: systolic arterial blood pressure changes during RBC transfusion. Time 0 corresponded to the end of baseline measurement. Time 10 was the end of Ringer's lactate solution transfusion. Time 20 corresponded to the end of hemorrhage. Time 40 was the end of transfusion of RBC and fluids and time 60 corresponded to the end of anesthesia. B: systolic arterial blood pressure changes during cell-free hemoglobin infusion. Time 0 corresponded to the end of baseline measurement. Time 20 was during cell-free hemoglobin transfusion. Time 40 corresponded to the end of hemoglobin transfusion. Time 60 corresponded to the end of anesthesia. Results are mean $\pm$ S.E.M. (n = 9). * P < 0.05 compared to the values of the same rats at the baseline. ^ P < 0.05 compared with the corresponding values of the control rats.

Fig. 2. Systolic arterial blood pressure and cerebral blood flow changes during hemorrhage and red blood cell (RBC) transfusion

Six-month old male Sprague–Dawley rats lost 20% total blood volume and then received RBC prepared from equal volume of the lost blood. A: systolic arterial blood pressure. B: rectal temperature. C: normalized left cerebral cortical blood flow. D: normalized right cerebral cortical blood flow. In all four panels, Time 0 corresponded to the end of baseline measurement. Time 10 was the end of Ringer's lactate solution transfusion. Time 20 corresponded to the end of hemorrhage. Time 40 was the end of transfusion of blood and fluids. Results are mean $\pm$ S.E.M. (n = 8). * $P < 0.05$ compared to the values of the same rats at the baseline. ^ $P < 0.05$ compared with the corresponding values in the control rats.

Fig. 3. Electrolyte and lactate concentrations in the stored red blood cell (RBC) solution and blood freshly drawn from rats after RBC transfusion

Six-month old male Sprague–Dawley rats lost 20% total blood volume and then received RBC prepared from equal volume of the lost blood. RBC solution just before being used for transfusion and blood freshly drawn from rats at the completion of the RBC transfusion were assayed. A: sodium concentrations. B: potassium concentrations. C: lactate concentrations. Results are mean $\pm$ S.E.M. (n = 8). * P < 0.05 compared to control rats. ^ P < 0.05 compared with RBC solution stored for 24 h.

Fig. 4. Arterial blood gases and hemoglobin results

Six-month old male Sprague–Dawley rats lost 20% total blood volume and then received red blood cells (RBC) prepared from equal volume of the lost blood. Blood freshly drawn from rats at the completion of the RBC transfusion was assayed. A: pH. B: PaCO₂. C: PaO₂. D: HCO₃⁻. E: base excess. F: blood hemoglobin. Results are mean ± S.E.M. (n = 8). * P < 0.05 compared to control rats.

Fig. 5. Performance in Barnes maze

Six-month old male Sprague–Dawley rats lost 20% total blood volume and then received red blood cells (RBC) prepared from equal volume of the lost blood. Rats were subjected to Barnes maze from one week after the transfusion. A: training sessions. Results are mean $\pm$ S.E.M. (n = 9). * P < 0.05 compared to the values of the same rats on day 1. B: one day after the training sessions. C: eight days after the training sessions. Results are mean $\pm$ S.E.M. (n = 9). ^ P < 0.05 compared to control rats. # P < 0.05 compared with rats transfused with old RBC.

Fig. 6. Performance in fear conditioning

Six-month old male Sprague–Dawley rats lost 20% total blood volume and then received red blood cells (RBC) prepared from equal volume of the lost blood. Rats were subjected to fear conditioning testing three weeks after the transfusion. A: context-related fear conditioning. B: tone-related fear conditioning. Results are mean $\pm$ S.E.M. (n = 9). * P < 0.05 compared to control rats.

Fig. 7. Expression of interleukin (IL) 6 and IL-1β in the cerebral cortex and hippocampus
Six-month old male Sprague–Dawley rats lost 20% total blood volume and then received red blood cells (RBC) prepared from equal volume of the lost blood. Brain was harvested at 24 h after the transfusion. A: interleukin 6 in the cerebral cortex. B: interleukin 6 in the hippocampus. C: interleukin 1β in the cerebral cortex. C: interleukin 1β in the hippocampus. Results are mean ± S.E.M. (n = 6). * P < 0.05 compared to control rats. ^ P < 0.05 compared with rats transfused with old RBC.

Fig. 8. Expression of ionized calcium-binding adapter molecule 1 (Iba-1) in the cerebral cortex and hippocampus

Six-month old male Sprague–Dawley rats lost 20% total blood volume and then received red blood cells (RBC) prepared from equal volume of the lost blood. Brain was harvested at 24 h after the transfusion. A: representative images. Iba-1 staining is in green color and Hoechst 33342 staining is in blue color. Scale bar = 200 μ m. The insert in the CA1 image after transfusion of old RBC was a high magnification image showing the body shape of a cell with positive staining for Iba-1. B: quantification data of Iba-1 staining in the cerebral cortex. C: quantification data of Iba-1 staining in the CA1. D: quantification data of Iba-1 staining in the CA3. E: quantification data of Iba-1 staining in the dentate gyrus (DG). Results are mean $\pm$ S.E.M. (n = 6). * $P < 0.05$ compared to control rats. ^ $P < 0.05$ compared with rats transfused with old RBC.

Fig. 9. Cell-free hemoglobin-induced impairment of learning and memory

Six-month old male Sprague–Dawley rats received intravenous 200 mg cell-free hemoglobin infusion. They were subjected to Barnes maze and fear conditioning tests from one week after the infusion. A: training sessions of Barnes maze test. B: memory phase of Barnes maze test. C: fear conditioning. Results are mean $\pm$ S.E.M. ($n = 9$). In panel A, * $P < 0.05$ compared to the values of the same rats on day 1. In panel B, ^ $P < 0.05$ compared to control rats, # $P < 0.05$ compared with rats transfused with cell-free hemoglobin.

Fig. 10. Expression of ionized calcium-binding adapter molecule 1 (Iba-1), interleukin (IL) 6 and IL-1 β in the cerebral cortex and hippocampus after receiving cell-free hemoglobin
Six-month old male Sprague–Dawley rats received intravenous 200 mg cell-free hemoglobin infusion. Brain was harvested at 24 h after the infusion. A: representative images. Iba-1 staining is in green color and Hoechst 33342 staining is in blue color. Scale bar = 200 μ m. B: quantification data of Iba-1 staining in the cerebral cortex and hippocampus. C: IL-6 levels in the cerebral cortex and hippocampus. D: IL-1 β in the cerebral cortex and hippocampus. Results are mean $\pm$ S.E.M. (n = 6). [^] P < 0.05 compared to control rats. [#] P < 0.05 compared with rats transfused with cell-free hemoglobin. DG: dentate gyrus.