This is the pre-peer reviewed version which has been published in final form at Artificial Cells, Nanomedicine, and Biotechnology, 46:7, 1349-1363

Long term safety and immunological effects of a nanobiotherapeutic , bovine poly-[hemoglobin-catalase-superoxide dismutase-carbonic anhydrase], after four weekly 5% blood volume top-loading followed by a challenge of 30% exchange transfusion.

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Support of this research: From the Canadian Blood Service (CBS) and the Canadian Institutes of Health Research (CIHR) to Professor TMS Chang.

Running head: Safety and immunology of Poly-[Hb-CAT-SOD-CA] . a nanobiotherapeutic

KEYWORDS:
Hemorrhagic shock, biotherapeutic, nanobiotherapeutic, nanomedicine, oxygen carrier, polyhemoglobin, carbon dioxide carrier, carbonic anhydrase, antioxidant, oxygen radicals, superoxide dismutase, catalase, artificial cells. long-term safety, top-loading, challenge, anaphylactic reaction, histamine, tryptase, antibody, immunology, transfusion, blood substitute.

Abstract

The long term safety and immunological effects of bovine poly-[hemoglobin-catalase-superoxide dismutase-carbonic anhydrase] in rats are studied by four-weekly 5% blood volume top-loading infusions followed by 30% blood volume exchange transfusion. There is no significant differences in growth, biochemistry and blood pressure between the control group receiving lactated ringer solution and those receiving the bovine poly-[hemoglobin-catalase-superoxide dismutase-carbonic anhydrase] There is no significant change in mean arterial pressures (MAP) before and after each weekly top-loading infusion.. After both the 4 weekly top-loading and the 30% exchange transfusions, the following safety and immune response evaluations are carried out. These include general studies on Ouchterlony double diffusion, total IgG and IgM, and complement activation. This is followed by quantitative measurements of specific antibodies against each of the following bovine components: Hb, CAT, SOD and CA in bovine poly-[hemoglobin-catalase-superoxide dismutase-carbonic anhydrase] . After the four weekly top-loading, the each rat received a challenge of 30% blood volume exchange transfusion. The MAP, histamine and tryptase levels are tested before and after the 30% exchange transfusion. There are no anaphylactic reactions as shown by the MAP or histamine and tryptase. The results showed no safety problem nor adverse immune responses. All the rats survived when followed for one week after the 30% exchange transfusion.

Introduction

Serious research on blood substitutes only started after the HIV contaminated donor blood crisis in 1989. Nowadays, the worldwide demand and lack of supply of donor blood, as well as natural or man-made disasters or epidemics in some regions, resulted in an urgent need to develop blood substitutes (Chang 2007, 2009, 2012, 2017, Kim & Greenburg. 2014, Mer et al, 2016, Weiskopf et al 2017). The first generation of blood substitute is hemoglobin-based oxygen carrier (HBOC). One of these is in the form of polyhemoglobin (polyHb) prepared by crosslinking Hb with diacid (Chang 1964) or glutaraldehyde

(Chang 1971). PolyHb has since been developed independently by many groups.-For example, glutaraldehyde crosslinked bovine polyHb developed by Biopure has undergone extensive clinical trials (Jahr, et al. 2008) and already approved for routine clinical use in South Africa (Mer, et al. 2016). Another group from Northfield has developed glutaraldehyde crosslinked human polyHb and has carried out extensive clinical trials on ambulance hemorrhagic shock patients in the U.S. (Moore, et al. 2009). Their result shows that human polyHb can delay the need for blood transfusion by 12 hours, while the saline control group requires blood transfusion within 1 hour (Moore, et al. 2009). However, there are 3% non-fatal cardiac side-effects in the polyHb group compared with 0.6% in the control saline group. The investigators suggest that it has potential for use in fatal hemorrhagic shock when donor blood is not available (Moore, et al. 2009). FDA did not approve this for clinical use, even though a number of investigators suggest that the risk/benefit ratios of polyHb have been shown in these emergency situations where donor blood is not available (Jahr, et al. 2008, Moore, et al. 2009, Weiskopf and Silverman 2013, Weiskopf, Beliaev, et al. 2017). .

For further improvement, the second generation blood substitutes has been developed in the form of Poly-[hemoglobin-catalase-superoxide dismutase-carbonic anhydrase] or Poly-[Hb-CAT-SOD]-with antioxidant functions (D'Agnillo and Chang 1998, Powanda and Chang 2001). This is important for ischemic condition including hemorrhagic shock to prevent ischemia reperfusion injuries (Alayash, 2004) by removing oxygen radicals during transfusion (Alayash, D'Agnillo and Buehler 2007). To enhance the antioxidant function, extra CAT and SOD enzymes are added to stroma-free hemoglobin (SFHb) for the development of poly-[Hb-SOD-CAT] which had been shown to prevent ischemia-reperfusion injury in a hemorrhagic shock-stroke rat model (Powanda and Chang 2001). Hsia's group later extended this by using PEG-hemoglobin-antioxidant enzyme mimetics (Buehler, et al. 2004, Ma and Hsia 2013).

Researchers find that the mortality and the severity of myocardial ischemia in hemorrhagic shock are related to elevated intracellular pCO_2 (Sims, et al. 2001). Therefore, the third generation of blood substitute includes the addition of carbonic anhydrase (CA) to produce poly-[hemoglobin-catalase-superoxide dismutase-carbonic anhydrase] (poly-[Hb-CAT-SOD-CA]) (Bian, Rong and Chang 2011). This fulfills all three major functions of red blood cells as O_2 and CO_2 carrier and oxygen radical remover. Poly-[Hb-CAT-SOD-CA] with enhanced enzyme activities has been tested in a rat model of 90-min hemorrhagic shock with loss of 2/3 of blood volume. The result shows that poly-[Hb-CAT-SOD-CA] with enhanced enzyme activities is more effective than whole blood in lowering the elevated intracellular pCO_2, improve cardiac recovery and avoid histological changes in liver, kidney, heart and intestine (Bian and Chang 2015).

Stroma-free haemoglobin (SFHb), is prepared from the content of red blood cells with the removal of lipid membrane. SFHb contains Hb and all the red blood cell enzymes, including CAT, SOD and CA. To enhance the red blood cell enzymes, commercial CAT, SOD and CA are added for the preparation of poly-[Hb-CAT-SOD-CA] (Bian, Rong and Chang 2011, Bian, Gao and Chang 2013, Bian and Chang 2015, Bian, Guo and Chang 2016). For avoiding the high cost of commercial enzymes, a cost-effective novel enzyme extraction method has been established to obtain these three enzymes from SFHb simultaneously (Guo, Gynn and Chang 2015). This avoids the need for expensive commercial enzymes and allows the large-scale production of poly-[Hb-CAT-SOD-CA] in the future.

This paper studies the long-term safety and immune response to bovine sourced poly-[Hb-CAT-SOD-CA] (heterologous) in rats, using 4 weekly top loading followed by a challenge with a larger volume (Figure 1).

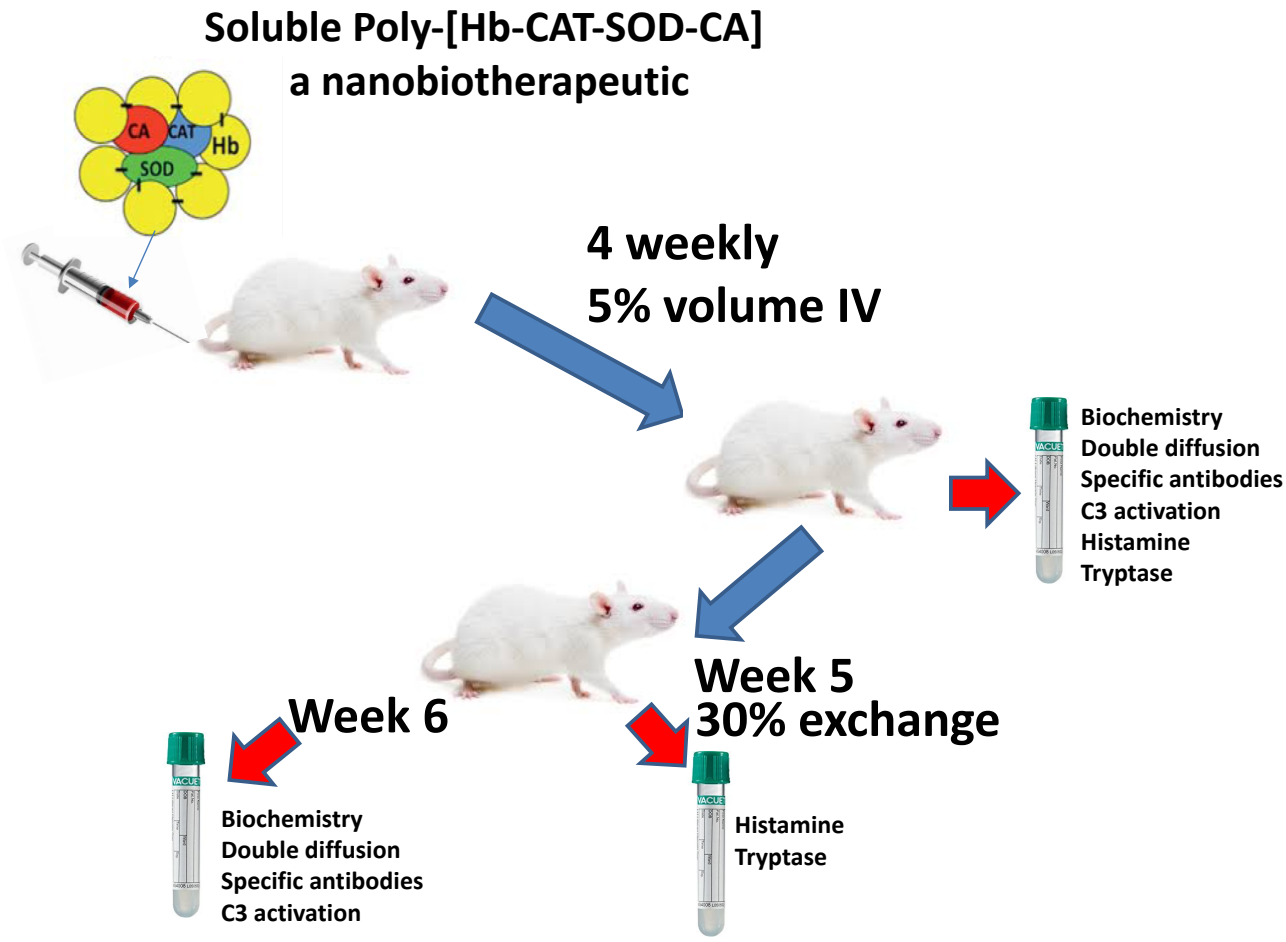

Figure 1: The rats are divided into 3 groups each receives 4 weekly infusion of one of the following samples: lactated ringer's solution (LR) as control group, bovine polySFHb (Phe) and bovine poly-[Hb-CAT-SOD-CA] (PHE) with enhanced enzyme activity. This is followed on week 5 by a 30% exchange infusion

The rats are divided into 3 groups and reach received one of the following samples: lactated ringer's solution (LR) as control group, bovine polySFHb (Phe) and bovine poly-[Hb-CAT-SOD-CA] (PHE) with enhanced enzyme activity. Body weight, plasma biochemistry heart rate and mean blood pressure are tested during the four-weekly top-loading infusion period. Mean arterial pressures (MAP) are measured before and after each 4 weekly top-loading. Immune study includes total IgG and IgM levels, Ouchterlony double diffusion, specific antibodies against bovine Hb, CAT, SOD and CA, and complement fragment 3a (C3a) activation test. After the 4 weekly Top-loading, each rat is challenged with a larger 30% blood volume by exchange transfusion using one of control (LR), bovine Phe or bovine PHE. The MAP, total histamine and tryptase levels before and after transfusion are analyzed for possible anaphylactic reactions. There are no significant adverse response and no immunological reaction in rats against bovine sourced poly-[Hb-CAT-SOD-CA] .

Methods and Materials

Materials

Vivaspin 20 Centrifugal Filter with both 10 kDa cut-off polyethersulfone (PES) membrane (VS2002) and 300 kDa cut-off PES membrane (VS2052) are purchased from Sartorious Stedium Biotech (Germany). All other experimental and analytical chemical reagents are purchased from Sigma. Sprague-Dawley male rats are purchased from Charles River. Mouse and Rat Tail Cuff Blood Pressure

(MRBP) system is purchased from IITC Life Science Inc. Enzyme-linked immunosorbent assay (ELISA) kits are purchased from different companies as indicated bellowed.

Bovine stroma-free hemoglobin solution preparation

Stroma-free hemoglobin (SFHb) solution is the red blood cell content with the removal of lipid membrane by organic solvent. To separate red blood cells, the heparinized bovine whole blood is centrifuged at 4500g at 4°C for 30 minutes. Remove the plasma layer carefully by vacuum suction using pasteur pipet. Use ice-cold lactated ringer's solution to ish the red blood cells thoroughly then centrifuge (4500g*30min) again to remove the plasma residue. Repeat the ishing steps for two more times. After removing the supernatant, we collect the red blood cells then adding 2 volumes cold lysis buffer (15mM sodium phosphate, pH 7.4) to the red blood cells. Mix the lysis buffer and the cells and incubate the mixture at 4°C for 30 minutes. Transfer the lyzed red blood cells to funnels and mixed with half volume of cold toluene thoroughly. Place the funnels in the fridge at 4°C overnight. Discard the upper organic layer, then centrifuge the hemolysate at 16000g for 2 hours. Evacuate the extra toluene by vacuum on ice for 1 hour. Dialyze each volume of SFHb against 20 volumes of lactated ringer's solution (pH 7.4) at 4°C overnight. Freeze the SFHb solution at -80°C for storage.

Bovine enzyme extraction and mix with bovine stroma-free hemoglobin solution

SFHb contains hemoglobin and red blood cell enzymes including CAT, SOD and CA. The enzyme extracted had been established previously (Guo, Gynn and Chang 2015). 300ml bovine SFHb (Hb concentration: 9g/dL) is placed in a beaker on ice. 60ml potassium phosphate buffer (3M, pH = 7.4) is added to bovine SFHb. Stir the mixture with medium speed for 5minutes on ice. Without stopping the stir, add drop-wise 120ml ice cold ethanol-chloroform solution with a speed of 1 drop/sec. Continue stirring for 30 minutes with medium speed on ice. Centrifuge solution 10mins at 5000rpm at 4°C. Collect supernatant layer (usually around 20ml) which contains SOD, CA, and CAT in > 80% recovery and Hb ≈ 0%. Transfer the supernatant to a new centrifugal tube, and centrifuge at 5000rpm for 10mins at 4°C to precipitate the suspending denatured Hb. Transfer 12 ml supernatant to each Vivaspin 20 centrifugal filter with10 kDa cut-off and centrifuge at 6000rpm at 4°C for 10 minutes. Add remaining supernatant to the same filters containing the enzyme solution and centrifuge at 6000rpm for 15 minutes at 4°C.Ish the extracted enzyme solution in the centrifugal unit with 5ml PBS (pH 7.4) in each filter to remove the remaining ethanol, mix the solution, and then centrifuge at 6000rpm at 4°C for 15 minutes. Repeat the ishing steps two more times. To minimize the final volume of concentrated enzymes, ish the enzymes solution with 2ml PBS (pH 7.4) in each filter and centrifuge at 6000rpm at 4°C for 15mins. Final volume of concentrated enzymes should be approximately 300µl in each filter.

Polymerization and purification

We crosslinked the SFHb with or without enzymes by glutaraldehyde to produce polySFHb or poly-[Hb-CAT-SOD-CA]. Mix 60ml bovine SFHb (Hb concentration: 7g/dL) with or without the concentrated enzymes that are prepared as described above. Add 1.5 ml 4M NaCl, 1.5 ml 3M potassium phosphate buffer (pH 7.4), 229 µl 2M lysine (200:1 lysine to hemoglobin molar ratio) and 0.84g trehalose to SFHb or SFHb + enzymes mixture. Shake the mixture at 160rpm at 4°C for 5mins. The frozen glutaraldehyde (0.5M) is thawed on ice water mixture by ultrasonic machine for 30mins. Mix glutaraldehyde evenly before use. Without stopping the shaker, add drop-wise 1.312 ml glutaraldehyde in 4 equal aliquots with intervals of 15mins. Keep shaking at 160rpm overnight at 4°C. Add 656µl 2M lysine (200:1 lysine to protein molar ratio) over 2 equal aliquots over 10 minutes and shake at 160 rpm at 4°C for 1 hour to quench. Centrifuge at 8000rpm at 4°C for 1 hour to remove any precipitate.

The sample is purified using Vivaspin 20 centrifugal filter with 300 kDa cut-off by giving positive pressure with centrifuge and lactated ringer's (LR, pH 7.4) solution to ish. After purification, the crosslinking product is dialyzed against lactated ringer's solution (v_{sample}/v_{LR}=1:20) 4°C overnight. The Hb concentration of the crosslinking sample is tested and adjusted to 7g/dL with LR solution. Freeze the samples at -80°C until use.

4 weekly small volumes (5% blood volume) Top-loading infusions and measurement of body weight, biochemistry, antibodies, heart rate and mean arterial pressure

The Animal Ethics Committee of McGill University has approved this experimental protocol (protocol number: 2001-3816). Sprague-Dawley rats, weighing 175 ± 20g, are randomly divided into 3 groups and each group contains 6 rats. The rats are intravenously injected every week for 4 weeks totally. All the samples are filtrated through 0.45μm filters before the Top-loading infusion. We placed the rat into a restrainer in Mouse and Rat Tail Cuff Blood Pressure (MRBP) system (IITC Life Science Inc.) and warmed it up (32°C) for 15 minutes. We then measured the heart rate and MAP with the system. After the measurement, we injected the samples through tail vein. The following groups are injected once a week for 4 weeks in total:

a. Control LR: Lactated ringer's solution, 1ml;
b. PHe: Bovine polySFHb (Hb: 7g/dL), 1ml;
c. PHE: Bovine poly-[Hb-CAT-SOD-CA] (Hb: 7g/dL), 1ml.

Each rat is injected with 1ml of sample per week, which simulates infusing 250ml per week for human for 4 weeks. Let the rat rest for 5 minutes in the system, then measured the MAP again. On the 5th week, rat plasma samples are collected from the rats through tail artery under anesthesia. Detail analytical procedures are described in the following part of plasma collection.

Larger challenge of 30% blood volume exchange transfusion after the above 4 weekly small volumes (5% blood volume) top-load

At week 6, the rats grew up to 400±25g. We randomly selected 4 out of 6 rats after Top-loading infusion, then performed 30% blood volume exchange transfusion on the 4 rats with the same sample (LR, Phe, or PHE) that is used for the Top-loading infusion. The rat is anesthetized by consistently inhalation of isoflurane (12ml/hr, oxygen flow: 2 L/min) in a sealed chamber. When the rat is in moderately deep plane of anesthesia, it is transferred to a ceramic plate with heating pad for keeping the body temperature of the rat during the blood exchange. Electro-probes are used for monitoring the ECG by MP150 data acquisition and analysis system with Acknowledge software (BIOPAC Systems, Inc). Isoflurane and oxygen are provided to rat via inhalation by mask throughout the whole procedure. Before the cannulation had been done, the isoflurane dose is 12ml/hr, oxygen flow: 2 L/min. A small incision about 1 cm is made on the skin at the left and the right femoral fossil region without cutting any tissue. Heparinized polyethylene catheters (PE10, Becton Dickinson and Company) are inserted into the left femoral artery and the right femoral artery and vein. The catheter in left artery is connected to the MP150 system (BIOPAC Systems, Inc) also for monitoring the MAP. The right femoral artery is cannulated for withdrawing the blood, while the right femoral vein is cannulated for infusion. After the catheters are placed in, isoflurane is adjusted to 6~9ml/hr, oxygen flow: 1~1.5 L/min). We allowed the MAP to be stabilized for 10 minutes after the adjustment of isoflurane/oxygen flow rate. After the stabilization, we withdraw 30% of the total blood volume (rat blood volume/rat body weight: 56ml/kg) with a speed of 0.5ml/min. Thus, we withdraw 7.5ml of whole blood within 15 minutes. 1ml of blood samples are collected for histamine and tryptase tests before infusion. We started to infuse the samples right after the bleeding within another 15 minutes. The samples for infusion are listed below. All the samples are filtrated through 0.45μm filters before the infusion. After infusion, the MAP is monitored for 30 minutes to determine if there is anaphylactic shock. Another 1ml of blood samples are collected after the 30 minutes observation for histamine and tryptase analysis.

a. Control LR: Lactated ringer's solution, 22.5ml (3 times volume of withdrawn sham blood);
b. Phe: Bovine polySFHb (Hb: 7g/dL), 7.5ml (same volume of sham blood);
c. PHE: Bovine poly-[Hb-CAT-SOD-CA] (Hb: 7g/dL), 7.5ml (same volume of sham blood).

At the end of the experiment, the catheters are removed, and arteries and vein are tied up with surgical polydioxanone suture (PDP304H, Ethicon) at the level of femoral fossil to allow for anastomosis blood flow. 0.1ml of lidocaine/bupivacaine is applied in the wound before closing the skin

with surgical sutures (PDP304H, Ethicon). After the wounds are sutured, 0.4ml of carprofen (10 mg/kg/day) is injected right away subcutaneously, and for the following two days thereafter. The rats are recovered from the 30% volume challenge and observed for one week.

Plasma collection

At week 5 and 7, after the rats are anesthetized by consistently inhalation of isoflurane (12ml/hr, oxygen flow: 2 L/min). 2ml blood is collected through tail artery from each rat into lithium heparin tube. The whole blood samples are centrifuged at 1500rpm, 4°C for 30 minutes. Plasma is collected and stored at -80°C until used.

Biochemistry test

The plasma biochemistry has been tested after four-week Top-loading infusion period and one-week recovery from blood exchange transfusion respectively to evaluate the safety of the samples to the rats with repeated injection. All the plasma samples are sent to the Animal Resources Centre at McGill University for the total panel test.

Quantification of total IgG and IgM levels in rats

To investigate the immune response to the samples, we tested the total IgG and IgM level in the rat plasma after four-week Top-loading infusion period and one week after 30% blood volume exchange transfusion respectively. The total IgG and total IgM are tested by the ELISA kits (Rat IgG total eBioscience 88-50490, Rat IgM eBioscience 88-50540). The testing methods are the same as instruction of the ELISA kits. Briefly, coat the ELISA plate with capture antibody (100µl/well) in coating buffer, incubate overnight at 4℃. Then block the plate with blocking buffer (250µl/well) at room temperature for 2 hrs or at 4°C overnight. After ishing the plates, adding 100µl/well of standard samples with 2-fold serial dilution or test plasma samples onto the plates, incubate for 2 hours at room temperature. Add the detection antibody (100µl/well) into the plates. Finally, add 100µl/well of substrate solution (TMB) and stopping solution and read the plates at 450nm and 570nm. The values of 570nm are subtracted from those of 450nm for data analysis.

Ouchterlony double diffusion

In order to detect the antibodies that may be produced to against the Phe or PHE, we did the double diffusion test on the rat plasma samples. The method of Ouchterlony double diffusion has been described elsewhere (Bailley 1996). Agarose is dissolved in the PBS (pH7.2) to 1% by heating in a microwave. The agarose solution (10ml) is poured to a depth of 5mm into Petri dishes (diameter of 5cm). The gels are allowed to cool down (for about 15min) and form gel at room temperature. Punch five holes (diameter of 7mm) on each gel. Plasma samples from rats are loaded in the middle wells, while the other four wells are filled with 50µl of the corresponding antigens (control LR, PHe and PHE) with serial concentrations: 0, 0.001g/dL, 0.01g/dL and 0.1g/dL in the dilution of PBS (pH7.4). The gel plates are then incubated at room temperature for 24h.

Quantification of specific antibody against bovine hemoglobin, catalase, superoxide dismutase and carbonic anhydrase

To determine more specifically the antibodies-against specific components in our poly-[Hb-CAT-SOD-CA], the ELISA tests for bovine Hb, CAT, SOD, and CA had been done on the rat plasma after four-week Top-loading infusion period and one-week after 30% blood volume exchange transfusion. Antigen coatings are prepared by loading the solutions of pure enzymes of SOD (Sigma-Aldrich), CAT (Sigma-Aldrich), CA (Sigma-Aldrich) and Hb (Sigma-Aldrich) on the plates for fluorescent ELISA. Antigen solutions (10 µg/ml) are prepared in carbonate-bicarbonate buffer (pH9.6). 0.2 ml of the above solutions is pipetted into each well of the microtiter plate, followed by incubation (covered) at room temperature for 2 hours. After incubation, the coating solution is removed, and ished three times by

PBST. 0.2 ml Aqua block buffer (Abcam) is loaded into each well and incubate at room temperature for 2 hours.

To make the standard curve, monoclonal primary antibodies against bovine SOD (Abcam), CAT (Abcam), CA (Abcam), and Hb (Santa Cruz Biotech) are prepared in the buffer for Immunosorbent Assay (PBST and Aqua block buffer v/v 1:4). The primary antibodies started with the dilutions of 1:10,000 (SOD), 1:10,000 (CAT), and 1:100,000 (CA) and 1:50 (Hb). To test the antibody concentration in the plasma samples, the plasma samples are diluted by 1:3000 into dilution buffer. After the blocking procedure, plates are ished three times with PBST and 0.2 ml of the diluted monoclonal antibody is added to each well, and Incubated at 4℃ overnight.

In the next day, plates are ished with PBST five times, and the secondary antibodies (Alexa Fluor®488, Abcam) against to the primary antibodies are prepared to be diluted 10,000 times with buffer for immunosorbent assay. 0.2 ml of this solution is added to each well, and incubated at room temperature for 2 hours. After incubation, plates are also ished with PBST five times, and ensure there is no bubble remaining in each well. Absorbance is detected directly in a fluorescent microplate reader (Ex: 495nm, Em: 519nm).

Complement activation

C3a levels in rat plasma samples are tested after four-week's Top-loading infusion period and one-week after 30% blood volume exchange transfusion respectively. As an important anaphylatoxin in the immune response (Bajic, et al. 2013), it is produced in the complement activation cascade, via the cleavage of complement fragment 3 (C3) by C3 convertase. There are three pathways to activate the complement: classical pathway, alternative pathway and mannose binding lectin (MBL) pathway. They all participate in the activation of C3 followed by the formation of C3a. Complement activation has been shown to play an important role in many adverse reactions to modified hemoglobin (Ning and Chang 1987). With better sensitivity than C3, C3a is an indicator to monitor the complement activation (Ning and Chang 1990).

The C3a level is determined by the ELISA kit (Cloud-Clone Corp. SEA387Ra), based on the procedure of complement activation test in Chang 1997 (Chang 1997) with slight changes. 0.1ml of samples (control LR, PHe, or PHE) are mixed with 0.4ml of corresponding plasma at 37°C at 60rpm for 1 hour. The reaction is quenched by transferring 0.4ml of the plasma-sample mixture into 2ml EDTA tubes to obtain the test samples. The method of ELISA test is the same as instruction of the kit. Add 100µl/well of standard samples with 2-fold serial dilution and 20-fold diluted test samples on the ELISA plates provided. 100 µl of biotin-antibody is then added to each well, followed by adding HRP-avidin (100µl/well). Finally, TMB substrate (90µl/well) and stopping solution (50µl/well) are applied. The concentrations of the samples are determined by their optical density under 450nm wavelength. The values of 570nm are subtracted from those of 450nm for data analysis.

Additional analysis for potential anaphylaxis with the 30% blood volume challenge

(1) Quantification of histamine levels in rat plasma

Histamine is released by mast cells that are activated by IgE. The important inflammatory mediator, histamine, acts on the vessel dilation, smooth muscle constriction and inflammation which lead to anaphylactic shock or other allergy symptoms such as rash and asthma (Amin, 2012). Histamine levels are tested before and 30 minutes after the infusion of samples (control LR, PHe, PHE). The procedure is the same as the instruction of the ELISA kit (ab213975, Abcam). Add 100µl of assay buffer to the B_0 (0 ng/ml standard) wells and 150 µl of the same assay buffer to the NSB wells on the ELISA plate provided. Pipette 100µl/well of standard histamine with 2-fold serial dilution and plasma samples. Add 50µl/well of histamine tracer except for the blank, and 50µl/well of histamine antibody to all wells except for the NSB and blank. Incubate the plate at room temperature for 1 hour. Use ishing solution (~400µl/well) to ish the ELISA plate for three ishes in total. Add 200µl/well of detection antibody into the wells except the blank. Seal and incubate the plate for 30 minutes, followed by another three ishes.

Applied 200µl of TMB substrate and 50µl of stopping solution to each well. Determine the optical density at 450nm, subtracted by 570nm value.

(2) Quantification of tryptase levels in rat plasma

Tryptase is an enzyme released from mast cells when they are activated during the allergic responses. Hence, tryptase has been used as a diagnostic marker in anaphylaxis and mastocytosis (Sperr, et al. 2002). Tryptase levels are tested before and 30 minutes after the infusion of samples (control LR, PHe, PHE). The procedure is the same as the instruction of the ELISA kit (E02M0256, BlueGene). Add 100µl of standards or plasma samples to the appropriate wells. Add 100µl of PBS (pH 7.0-7.2) in the blank control wells. Add 50µl of conjugate solution to each well except the blank control wells and mixing the wells thoroughly. Cover and incubate the plate for 1 hour at 37°C. Ish the wells with ishing buffer for 5 times. Add 50µl substrate A and 50µl substrate B to each well including blank control well. Cover and incubate the plate for 10 minutes at 37°C in the dark place. Add 50µl stop solution to all the wells and determine the optical density at 450nm using microplate reader.

Statistics

Data are presented in a way of mean ± standard deviation (SD). Difference among the control groups (lactated ringer's solution, LR) and bovine PHe and PHE groups are assessed by one-way ANOVA. To determine the relationship between the MAP and the week of injection, and the MAP changes during the 30 minutes observation period in the exchange transfusion, the correlation coefficients and its p-values by two-sided are tested, with methods of pearson, kendall and spearman. The confident level is 95% in all tests.

Results

Effects on body weight

Figure 2 shows that the body weights of rats increase from 178±20g to 357±30g in 4 weeks. There are no significant differences from the control group of lactated Ringer.

Figure 2. Body weight changes of the rats during the four-week Top-loading infusion period. The body weight is measured in gram (g). Each figure shows a group of rats injected with corresponding sample (control LR, PHe, PHE). The body weight in rats injected with control (LR) are 158±3.7g, 227±7g, 288±17g, 348±20g. The body weight in rats injected with bovine PHe are 183±9g, 248±15g, 313±9g, 366±25g. The body weight in rats injected with bovine PHE are 198±4g, 257±5g, 318±9g, 367±9g. There are no significant differences from the control group

Effects on biochemistry

Biochemistry in rat plasma samples are tested after four-weekly 5% top-load and also one-week after 30% blood exchange transfusion (Table 1). For better interpretation and visualization of the results, some important indicators are selected for analysis in Figure 3-4.

The indicators of kidney function, blood urea nitrogen (BUN) and creatinine, showed no significant difference between the control (LR) groups and the bovine sample groups. Albumin, albumin/globulin ratio, alanine transaminase (ALT), aspartate aminotransferase (AST) and bilirubin are the indicators of liver function. There are no significant difference among the control groups and bovine blood substitute groups. The level of creatine kinase (CK) also showed no significant difference between the control (LR) groups and the bovine sample groups in CK levels.

Table 1. Biochemistry test.

		Lactacted ringer's		poly(SFHb)		poly(SFHb-CAT-SOD-CA)		Lactacted ringer's		poly(SFHb)		poly(SFHb-CAT-SOD-CA)	
		After 4 weekly IV		After 4 weekly IV		After 4 weekly IV		After 30% transfusion		After 30% transfusion		After 30% transfusion	
tests	Units	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Total Protein	g/L	57	1.52752523	57.3333333	1.1055416	58.8333333	1.06718737	57	2.23606798	60	0	60.75	0.8291562
Albumin	g/L	31.8333333	1.21335165	31.6666667	1.1055416	32.8333333	1.46249406	31.5	1.11803399	33	0.70710678	32.5	0.8660254
Albumin/Globulin ratio		1.25	0.05	1.25	0.05	1.25	0.07637626	1.225	0.08291562	1.25	0.05	1.15	0.08660254
Glucose	mmol/L	10.7333333	0.81785628	12.1666667	1.18133634	11.4	0.56273143	10.975	1.30838641	10.9	0.6670832	10	0.53851648
BUN Urea	mmol/L	5.71666667	0.63879226	4.86666667	0.74311656	5.2	0.76376262	4.725	0.25860201	4.8	0.41833001	4.775	1.29687123
Creatinine	μmol/L	17.8333333	0.372678	18.1666667	1.06718737	21.1666667	1.57233019	20.25	1.08972474	19.75	1.47901995	20.75	4.96865173
Total Bilirubin	μmol/L	4.33333333	1.80871659	4.33333333	1.13437475	3.16666667	0.89752747	3	0.70710678	3	0.70710678	4.75	1.29903811
ALT	U/L	50.5	6.92218655	45.6666667	6.62486897	53.5	2.5	45.5	4.55521679	39.5	3.04138127	45.75	3.49106001
AST	U/L	69.3333333	3.81517438	67.3333333	4.30761599	73.5	1.25830574	61.5	2.87228132	66.25	4.49305019	82	10.2469508
Alkaline Phosphatase	U/L	266.833333	29.1342677	228.666667	20.0305323	280.833333	33.3637361	192	19.9499373	181.75	12.2142335	203.5	41.8897362
CK	U/L	115.333333	12.9314432	151.333333	17.9412622	138.166667	27.9846188	96	10.7470926	116.25	5.62916512	135.75	18.6731759
Cholesterol	mmol/L	2.04833333	0.2663592	2.3	0.59205856	1.94833333	0.25458572	1.67	0.18881208	2.4325	0.24200981	1.9125	0.3647859
Sodium	mmol/L	135	3.05505046	141	2.94392029	145.666667	1.79505494	141.75	0.4330127	141.25	0.8291562	142.5	0.8660254
Potassium	mmol/L	10.0166667	0.90446424	4.58333333	0.26718699	4.88333333	0.22669118	4.6	0.45276926	4.95	0.28722813	5.225	0.21650635
Chloride	mmol/L	100	2.23606798	104	0.81649658	106	1	102.75	1.29903811	103.5	1.11803399	104.5	1.11803399
Calcium	mmol/L	2.51166667	0.05842849	2.65	0.05446712	2.70666667	0.04384315	2.595	0.025	2.665	0.04609772	2.72	0.01
Phosphorus	mmol/L	2.46333333	0.09012337	2.66666667	0.12762793	2.70833333	0.11964067	2.3725	0.0973075	2.385	0.22655022	2.5625	0.13479151
Magnesium	mmol/L	0.75333333	0.04606758	0.69333333	0.05088113	0.71833333	0.0157233	0.715	0.02598076	0.6825	0.02384848	0.6775	0.02277608

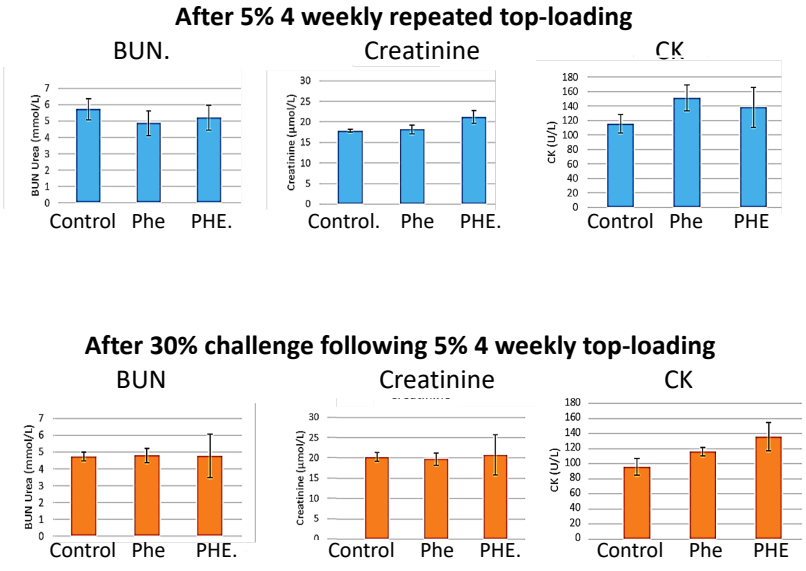

Figure 3. BUN urea, creatinine and CK levels. All data are in table 1. No significant statistical differences from the control groups

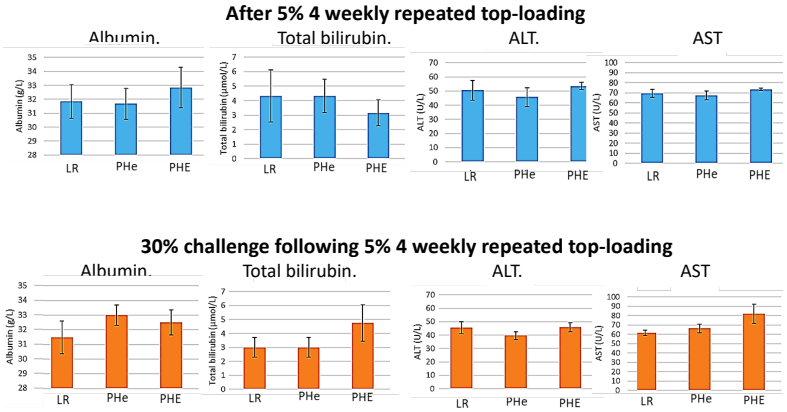

Figure 4. Albumin, total bilirubin, ALT and AST levels. All data are in table 1. No significant statistical differences from the control groups

Ouchterlony double diffusion

Ouchterlony double diffusion test on the rat plasma samples are conducted to detect any antibodies produced against the bovine poly-[Hb-CAT-SOD-CA] after four-weekly top-loading infusions and one-week after exchange transfusion. Plasma samples are in the middle wells of the plates. Antigens with different concentrations are added to the peripheral wells. The concentrations of the antigens in upper left, upper right, lower right and lower left wells are 0, 0.001g/dL, 0.01g/dL and 0.1g/dL respectively. These concentrations of antigens added to the wells gives a wide detectable range for the specific antibodies in plasma. If the plasma samples contain the antibodies that against the injected antigens, an antibody-antigen precipitation would present between the two wells. Otherwise, no precipitation can appear.

Figure 5 is the positive control of bovine serum albumin (BSA) showing BSA and anti-BSA antibody precipitation. Figure 6 for negative control, lactated Ringer, shows no precipitation line. Figure 6 shows no precipitation line with no antibodies against the bovine PolySFHb (PHe) or bovine poly-[Hb-CAT-SOD-CA] (PHE). After 30% blood volume exchange transfusion with control (LR), PHe or PHE there is also no antigen-antibody precipitation (Figure 7)

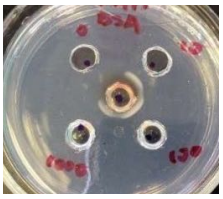

Figure 5 Ouchterlony double diffusion test. Positive control of BSA showing BSA and anti-BSA antibody precipitation

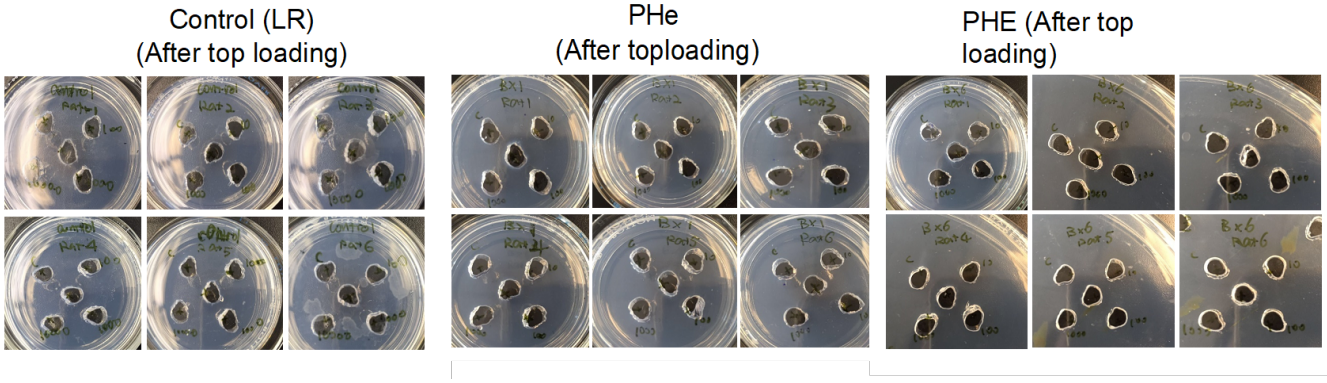

Figure 6 Ouchterlony double diffusion test on rats after 4 weekly 5% top-loading infusion with lactated ringer's solution as control, Bovine PHe or PHE (6 rats/group) showing no antigen-antibody precipitations

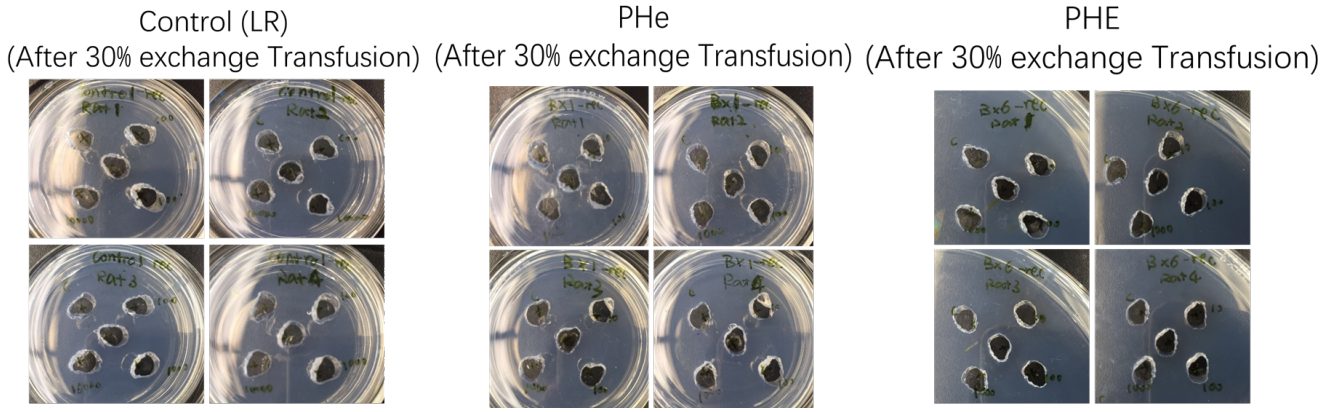

Figure 7 Ouchterlony double diffusion test on rats after 30% blood volume exchange transfusion with control (LR), PHe or PHE (4 rats/group)) showing no antigen-antibody precipitations

Effects on total IgG and IgM levels in rats

Total IgG and IgM levels are tested after 4 weekly 5% top-loading and after 30% blood volume exchange to study the immune response to bovine poly-[Hb-CAT-SOD-CA] in rats (Figure 8&9). For total IgG tests (Fig 8), there is no significant difference between the LR (control) group and the other two bovine samples groups. Total IgM levels (Fig 9) after top-loading infusion with LR control group, PHe or PHE are 0.56±0.33 mg/ml, 1.24±0.60 mg/ml and 2.28±0.87 mg/ml; p-value by one-way ANOVA is 0.002 (<0.05). Total IgM levels after 30% blood volume exchange transfusion with LR control group, PHe or PHE are 0.57±0.22 mg/ml, 1.79±0.75 mg/ml and 2.11±1.33 mg/ml; p-value by one-way ANOVA is 0.14 (>0.05). However, if we only compare the control group and PHe group by student's T-test, the p-value is 0.035 (<0.05); compare the control group and PHE by student's T-test, p-value is 0.093 (>0.05).

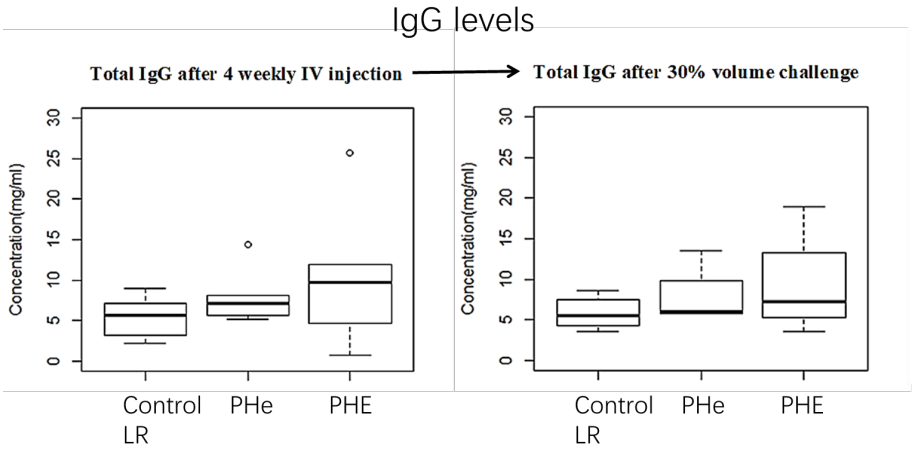

Figure 8 Total IgG levels after four-weekly 5% blood volume top-loading infusions followed by 30% blood volume exchange transfusion. After 4-weekly injection-period, the total IgG levels in rat plasma against LR control group, PHe and PHE are 5.42±2.36 mg/ml, 7.88±3.13 mg/ml and 10.41±7.80 mg/ml respectively; p-value by one-way ANOVA is 0.32 (>0.05). After 30% blood volume exchange transfusion, the total IgG concentrations in LR control group, PHe and PHE group are 5.84±1.86 mg/ml, 7.82±3.27 mg/ml and 9.24±5.80 mg/ml; p-value by one-way ANOVA is 0.60 (>0.05).

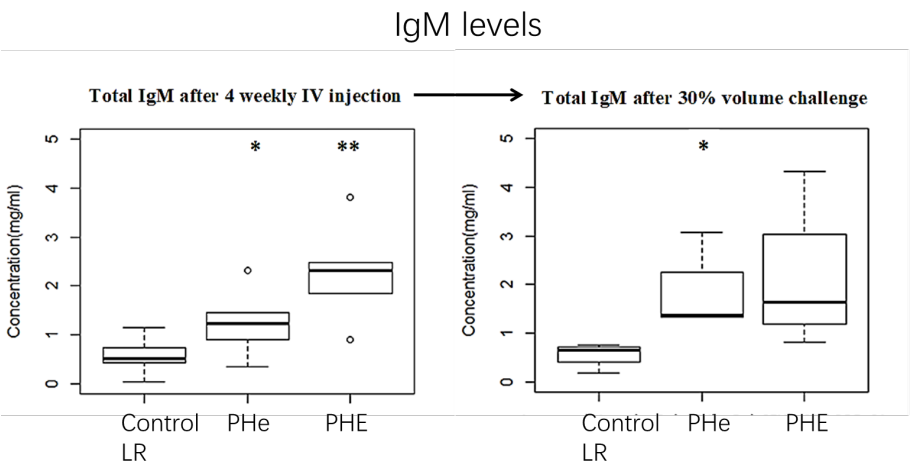

Figure 9 Total IgM levels after four-weekly 5% blood volume top-loading infusions followed by 30% blood volume exchange transfusion. Total IgM levels after top-loading infusion with LR control group, PHe or PHE are 0.56±0.33 mg/ml, 1.24±0.60 mg/ml and 2.28±0.87 mg/ml; p-value by one-way ANOVA is 0.002 (<0.05). Total IgM levels after 30% blood volume exchange transfusion with LR control group, PHe or PHE are 0.57±0.22 mg/ml, 1.79±0.75 mg/ml and 2.11±1.33 mg/ml; p-value by one-way ANOVA is 0.14 (>0.05). However, if we only compare the control group and PHe group by student's T-test, the p-value is 0.035 (<0.05); compare the control group and PHE by student's T-test, p-value is 0.093 (>0.05).

Effects on specific antibody against bovine hemoglobin, catalase, superoxide dismutase and carbonic anhydrase

IgG and IgM changes could be due to many other reasons. The above double diffusion studies show that there is no antibody produced to the poly-[Hb-CAT-SOD-CA] complex itself. For more specific analysis we carry out further studies on the potential antibody produced specifically against each component within bovine sourced poly-[Hb-CAT-SOD-CA], The excess of hemoglobin in the poly-[Hb-CAT-SOD-CA] complex nanoencapsulate the more antigenic enzymes and can prevent them from immunological response (Figure 10).

SPECIFIC ANTIBODIES TO
I. Complex: double diffusion
II. Individual component of complex

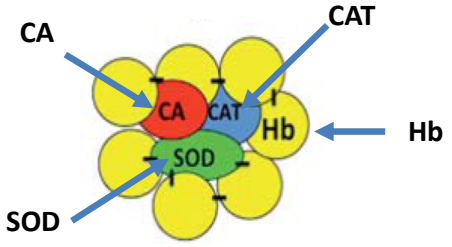

Figure 10: The excess hemoglobin in the poly-[Hb-CAT-SOD-CA] complex nanoencapsulate the more antigenic enzymes

Thus, specific antibodies against bovine Hb, CAT, SOD and CA are determined after: (1) 4 weekly top-loading infusions of LR, PHe and PHE; and (2) 4 weekly top-loading infusions of LR, PHe and PHE followed by 30% blood volume exchange transfusion. As we can see from figure 10~13, after 5% top-loading infusion and followed by 30% exchange transfusion, there is no antibodies against bovine CAT and CA. There is also no significant difference in antibody levels against bovine Hb and SOD among control group (LR) and sample groups (PHe and PHE). There is no antibody against CAT and CA after four weekly 5% top-loading followed by 30% volume challenge (Fig. 13, and 14). There is no significant difference between control (LR), PHe and PHE in the concentrations of antibodies against bovine Hb and SOD after 4-weekly injections and 30% volume challenge (Fig 11&12). The values of anti- bovine Hb antibodies are 0.41±0.09mg/ml, 0.49±0.13mg/ml and 0.42±0.08mg/ml respectively. The p-value by one-way ANOVA is 0.83 (> 0.05).-After 30% blood volume exchange, the antibodies against Hb in the group of control (LR), PHe and PHE are 0.44±0.14

mg/ml, 0.40±0.10 mg/ml and 0.52±0.11 mg/ml respectively; p-value by one-way ANOVA is 0.47 (> 0.05. The antibodies against SOD after 4-weekly top-loading in control (LR), PHe and PHE are 1.92±2.09 ng/ml, 1.30±2.11 ng/ml and 1.28±2.57ng/ml respectively. P-value by one-way ANOVA is 0.48 (> 0.05). The antibodies against bovine SOD control (LR), PHe and PHE are 21.13±7.57 ng/ml, 28.79±15.07 ng/ml and 6.80±7.79 ng/ml; p-value by one-way ANOVA is 0.09 (> 0.05)..

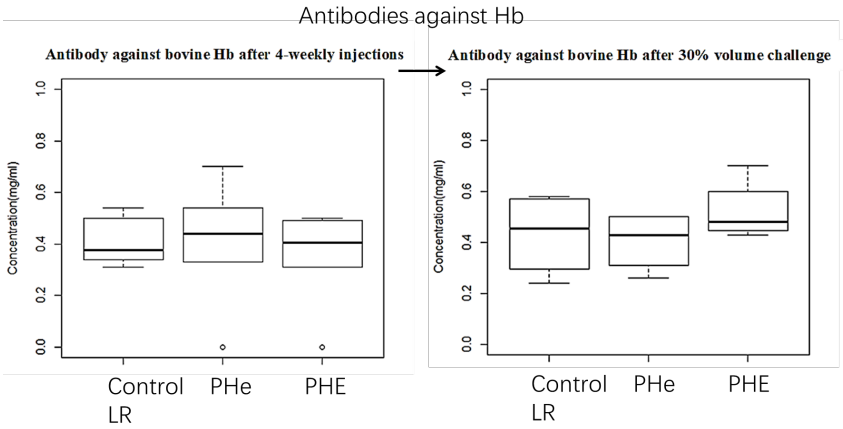

Figure 11 The concentration of antibodies against bovine Hb after 4-weekly injection in group of control (LR), PHe and PHE are 0.41±0.09mg/ml, 0.49±0.13mg/ml and 0.42±0.08mg/ml respectively. The p-value by one-way ANOVA is 0.83 (> 0.05). After 30% blood volume exchange, the antibodies against Hb in group of control (LR), PHe and PHE are 0.44±0.14 mg/ml, 0.40±0.10 mg/ml and 0.52±0.11 mg/ml respectively; p-value by one-way ANOVA is 0.47 (> 0.05).

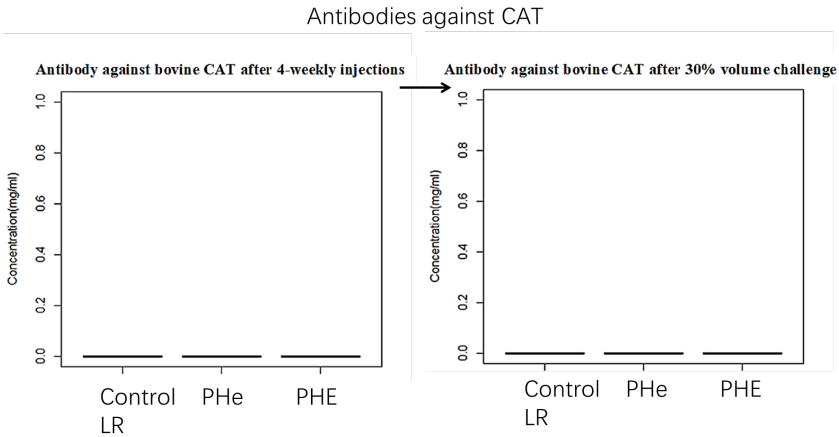

Figure 12 Antibody against bovine CAT after 4-weekly injections followed by 30% blood volume exchange transfusion. No antibody is detected.

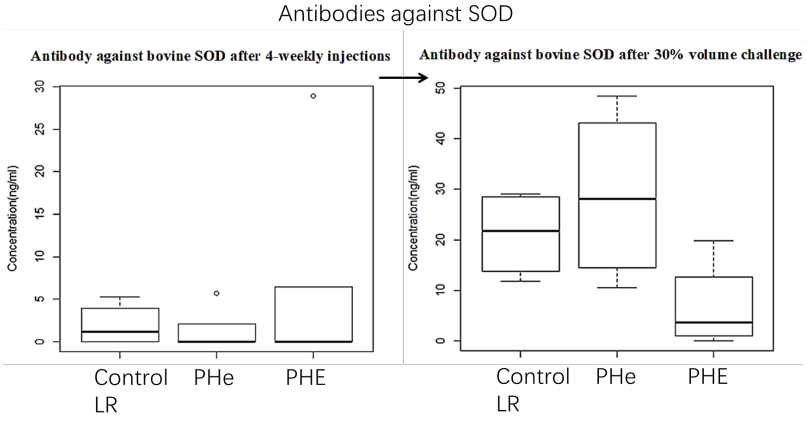

Figure 13 The antibodies levels against SOD after 4-weekly Top-loading infusions in group of control (LR), PHe and PHE are 1.92±2.09 ng/ml, 1.30±2.11 ng/ml and 1.28±2.57ng/ml respectively. P-value by one-way ANOVA is 0.48 (> 0.05). The antibodies against bovine SOD in group of control (LR), PHe and PHE are 21.13±7.57 ng/ml, 28.79±15.07 ng/ml and 6.80±7.79 ng/ml; p-value by one-way ANOVA is 0.09 (> 0.05).

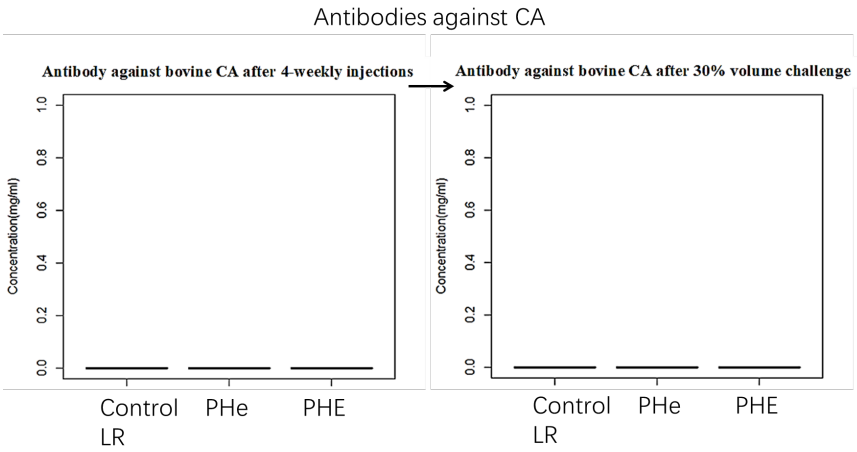

Figure 14 Antibody against bovine CA after 4-weekly injections followed by 30% blood volume exchange transfusion. No antibody is detected.

Effects on C3a activation

For more detailed evaluation of the immune response in terms of complement activation in rats, C3a levels after: (1) four-week 5% intravenous injections and (2) four-week t5% op-loading infusions followed by 30% blood volume exchange (Figure 15). In both cases, no significant difference can be seen in C3a levels between the LR control group and blood substitute groups.

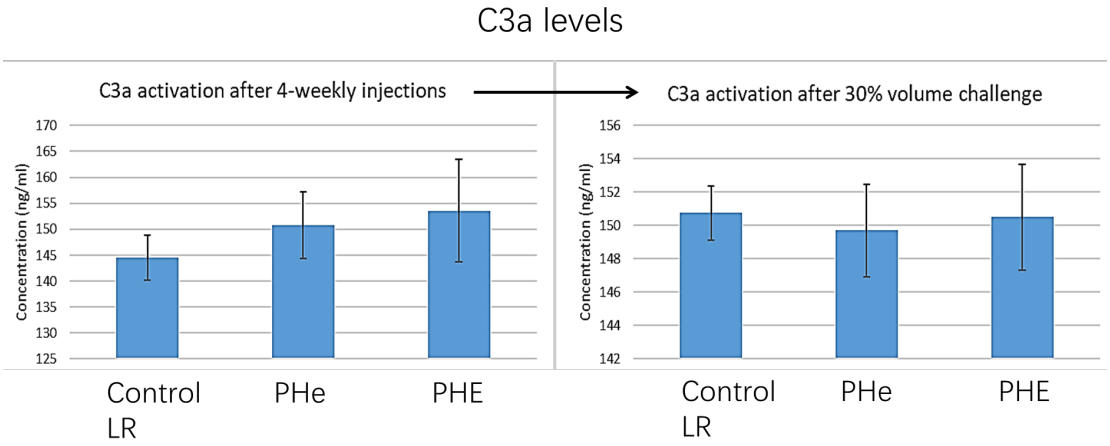

Figure 15. C3a activation test. After four-weekly injections. C3a levels in LR control group, PHe and PHE groups are 144.49±4.39 ng/ml, 150.74±6.38 ng/ml and 153.56±9.90 ng/ml respectively; p-value by one-way ANOVA is 0.16 (>0.05). After four-week top-loading infusions followed by 30% blood volume exchange. C3a levels in LR control group, PHe and PHE groups are 150.74±1.60 ng/ml, 149.70±2.77 ng/ml and 150.50±3.18 ng/ml respectively; p-value by one-way ANOVA is 0.88 (>0.05).

Effects on mean arterial pressure during each 4-weekly intravenous injection

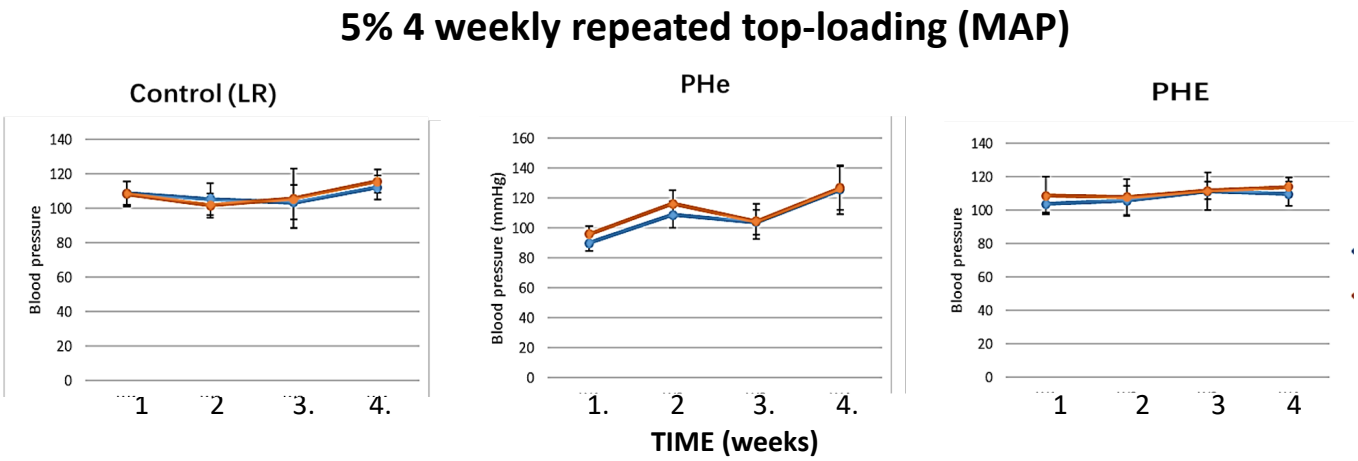

Figure 16. Mean arterial pressures are recorded just before and 5 minutes after each Top-loading infusion. Each figure shows a group of rats injected with corresponding sample. Control (LR): the correlation coefficients between the blood pressure before or after injection are 0. 27 and 0.56 respectively. The p-values of the coefficients are 0.73 and 0.44 respectively. PHe: the correlation coefficients between the blood pressure before or after injection are 0.89 and 0.77 respectively. The p-values of the coefficients are 0.11 and 0.23 respectively. PHE: the correlation coefficients between the blood pressure before or after injection are 0.16 and 0.30 respectively. The p-values of the coefficients are 0.24 and 0.25 respectively.

MAP is measured before and after each 4 weekly 5% Top-loading each week. No significant difference in MAP between before and after injection can be seen (Figure 16). The correlation test is performed by r program, and the p-values of all the correlation coefficients are > 0.05, thus we cannot reject the hypothesis that the correlation coefficients are 0.

Effect of 4 weekly 5% toploading followed by 30% blood volume exchange transfusion on mean arterial pressure

The MAP changes are monitored and recorded as shown in Figure 17.

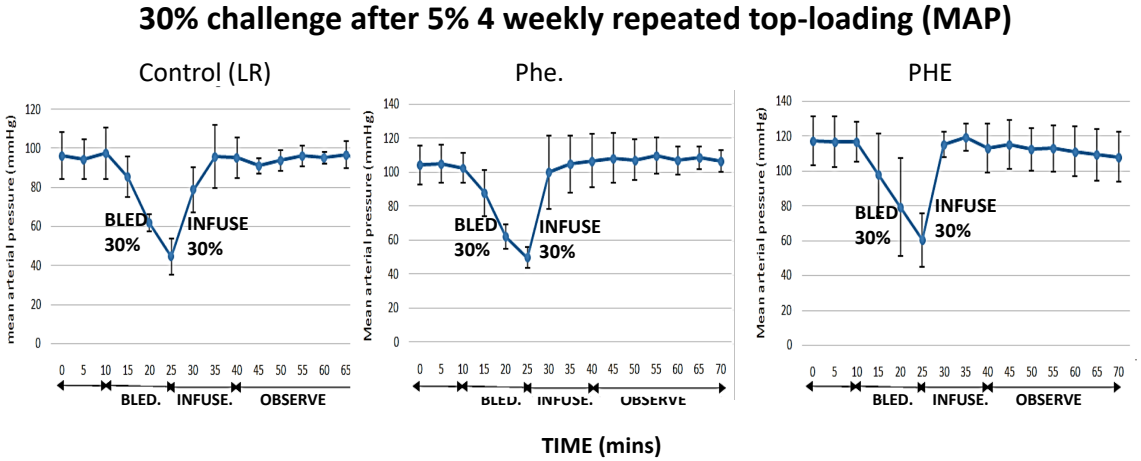

Figure 17. Mean arterial pressure during the 30% blood volume exchange transfusion. Each figure showed a group of rats infused with one of lactated ringer's solution (control), PHe, PHE. Control (LR): the correlation coefficients of the blood pressure before and after infusion is 0.13. The p-value of the coefficient is 0.55. PHe: the correlation coefficient between the blood pressure before or after

infusion is -0.03. The p-value of the coefficient is 0.87. PHE: the correlation coefficients between the blood pressure before or after infusion are -0.16. The p-value of the coefficient is 0.45. Thus, there are no significant changes

The first 10 minutes is the stabilization period. This is followed by 15 minutes of bleeding period with the removal of 30% of blood. After this, one of the samples (LR, PHe, PHE) is infused over 15 minutes. This is followed by 30 minutes of observation. There are no significant changes in MAP during the 30 minutes post-infusion period. Neither upward nor downward trend can be seen. The p-values of all the correlation coefficients for the MAP in the 30 minutes observation period are > 0.05, thus we cannot reject the hypothesis that the correlation coefficients are 0. Therefore, no anaphylactic shock is observed after 30% blood volume exchange transfusion. In order for a more detailed test of anaphylactic changes, plasma histamine and tryptase are also followed (Fig. 18, 19)

Additional specific analysis on the potential anaphylactic effect as shown by histamine levels and tryptase levels before and after the larger challenge of 30% exchange transfusion

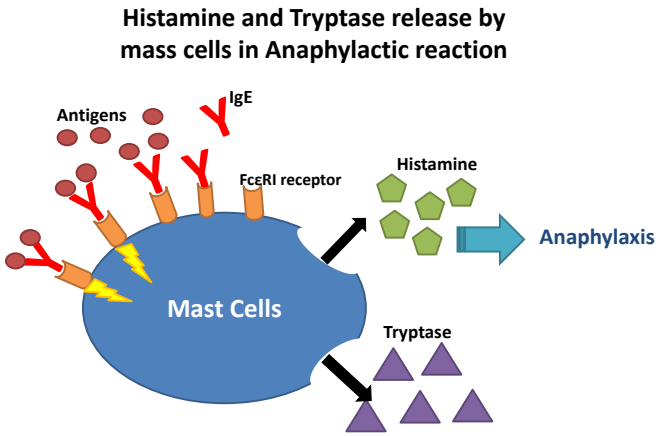

Figure 18: In anaphylactic reaction both Histamine and Tryptase are released from the mast cells

Release of histamine by mast cells is responsible for anaphylactic reaction (Figure 18). However, histamine is unstable and measurement of plasma histamine is not accurate. Tryptase is also released by the mast cell in anaphylactic reaction. It is more stable and as a result the measurement of plasma tryptase is more accurate.

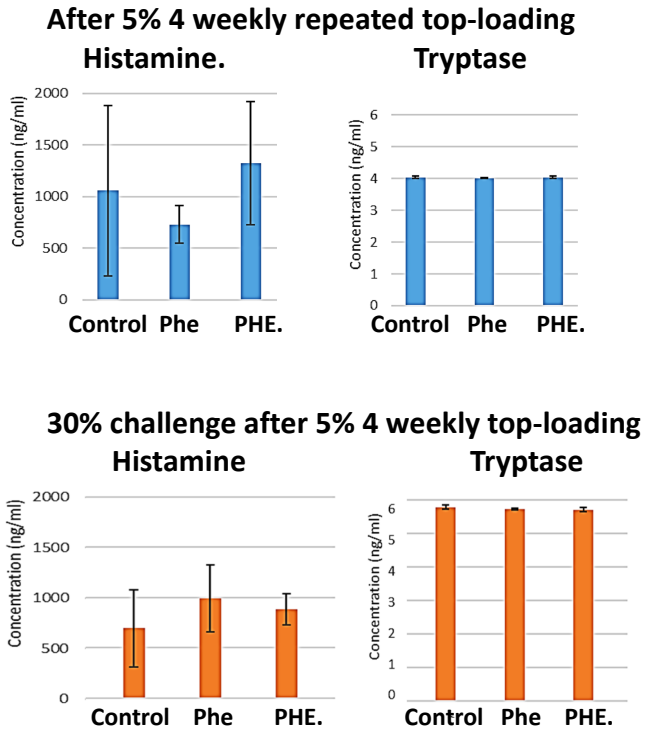

Figure 19. Histamine and tryptase levels tested before and 30 minutes after 30% blood volume exchange transfusion of LR (control), PHe or PHE. Plasma Trptase analysis is more accurate than histamine as shown by the higher SD for histamine. The histamine levels: the p-value by one-way ANOVA among the three groups is 0.62 (>0.05). The tryptase levels: comparing the LR control groups and the PHe and PHE groups, the p-value by one-way ANOVA is 0.51 (>0.05) before infusion, and the p-value of after infusion is 0.21 (>0.05). Thus, there is no significant changes in the histamine or tryptase levels between the control group and the PHe and PHE groups

To investigate more quantitatively whether there are any anaphylactic reactions after the larger challenge of 30% blood volume, histamine and tryptase levels are tested before and 30 minutes after infusion of samples (LR, PHe or PHE). There are no significant differences in histamine and tryptase analysis when comparing the LR control group and the bovine PolySFHb (PHe) and poly-[Hb-CAT-SOD-CA] (PHE) groups (Figure 19),

Histamine and tryptase levels are tested before and 30 minutes after 30% blood volume exchange transfusion of LR (control), PHe or PHE. The histamine levels in LR control group before and after infusion are 1058.62±825.06 ng/ml and 695.47±384.02 ng/ml. The histamine levels in PHe group before and after infusion are 730.37±182.28 ng/ml and 992.30±334.91 ng/ml. The histamine levels in PHE group before and after infusion are 1323.64±598.83 ng/ml and 882.72±156.77 ng/ml. The p-value by one-way ANOVA among the three groups is 0.62 (>0.05). The tryptase levels before infusion in LR, PHe and PHE groups are 4.04±0.03ng/ml, 4.01±0.01ng/ml, and 4.04±0.03ng/ml; while after infusion, the values are 5.79±0.06ng/ml for the LR control group, 5.73±0.02ng/ml for PHe, and 5.71±0.05ng/ml for PHE.. By comparing the LR control groups and the blood substitutes groups, the p-value by one-way ANOVA is 0.51 (>0.05) before infusion, and the p-value of after infusion is 0.21 (>0.05). Thus, there are no significant changes in the histamine or tryptase levels between the control group and the PHe and PHE groups.

Discussion

In the present paper we study the lng term safety and immunological effects of heterologous poly-[Hb-CAT-SOD-CA] (PHE) in rats after (1) four weekly 5% blood volume top-loading and (2) four weekly 5% blood volume top-loading followed by 30% blood volume exchange transfusion.

The rats in both the control group and bovine groups grew by 60±10g/week; and no significant difference between LR control group and PHe, PHE. There are no significant differences in the biochemistry between the control LR group and the bovine sample groups. The MAP before and after each of the 4 weekly 5% top-loading showed no significant changes; and there is no significant difference between the control group and PHe, PHE groups. These results suggest that there are no adverse effects in rats after 4 weekly intravenous injections.

The effect on immunological response is also studied. Ouchterlony double diffusion test is first performed as a screening test to see if there are any antibodies produced against the PHe, PHE complexes. There is no precipitate on the plates showing the absence of antibodies. This is followed by

another screening test on the total IgG and IgM and there are no significant changes detected in IgG ELISA test. However, the IgM concentrations after Top-loading infusions with bovine PolyHb-CAT-SOD-CA groups are higher than that in the control group ($p<0.05$). Since total IgG and IgM test are nonspecific and could be the result of other causes, we carried out more specific analysis by quantification of specific antibodies to the specific bovine components in PHe and PHE (Hb, CAT, SOD and CA). Specific antibodies against bovine CAT and CA are not detected; and no significant difference can be seen in the antibodies against bovine Hb or SOD between the control (LR) group and Phe, PHE. Moreover, no C3a activation is detected.

In order to rule out the possibility of anaphylactic reaction we carried out the following study. Four weekly 5% blood volume top-loading infusions of LR, PHe and PHE is followed by 30% blood volume exchange transfusion. The MAPs before and after each of the four weekly 5% toploading showed no significant changes. There is also no significant changes after the larger challenge of 30% blood exchange following the 4 weekly 5% toploading. There is also no significant alterations in histamine and tryptase levels before and after the 5% toploading and also the 30% challenge that followed this. When followed for 1 week, all the rats survive and in good health after the 30% volume exchange transfusion. Thus, no anaphylactic reactions or adverse immune response are observed.

In summary, no adverse long-term safety problems and mortality happened after the 4 weekly 5% volume top-load followed by the 30% volume challenge. This study shows the potential to use bovine bovine PolyHb-CAT-SOD-CA with enhanced red blood cell enzymes for transfusion.

Acknowledgements

This laboratory is not connected with any commercial organizations. The research support for the study reported here comes from the joint program of the Canadian Blood Service/Canadian and the Institutes of Health Research that requires the authors to state that: “The opinions expressed in this article are the opinions of the investigators of this paper and not necessarily those of the Canadian Government nor of the Canada Blood Service”.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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