28.2.3 Animal Preparation and Instrumentation

The animals, pre-medicated with atropine sulfate (0.07 mg/kg i.m.). Anesthesia was induced by intramuscular injection of ketamine hydrochloride (5 mg/kg i.m.). Animals were orally intubated, inhalation anesthesia was maintained with 2.0–2.5 %–sevoflurane mixed air supplied by an anesthesia apparatus (SN–487, Shinano Seisakusho Co., Tokyo, Japan). The concentration of sevoflurane (2.0–2.5 %) was adjusted as necessary to maintain the animal at a stable plane of anesthesia. Visual monitoring of spontaneous respiration was performed.

Electrocardiogram (EKG) electrodes were attached to the feet. A 5.5-F Thermodilution catheter (631Hf55; Edwards Lifescience, Irvine, CA, USA) was placed in the pulmonary artery via the right femoral vein for measurements of the mean pulmonary arterial pressure (MPAP), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), and cardiac output. The left femoral artery was cannulated to monitor arterial pressure as well as for blood sampling. The pressure line was connected to transducers (5100TW; Edwards Lifescience, Irvine, CA, USA), and these transducers and the EKG line were connected to a polygraph system (LEG-1000, Nihon Kohden Co., Tokyo, Japan). The right femoral artery was cannulated with a 16G I.V. cathter (Angiocath; Becton–Dickinson, Sandy, Utah, USA) to control the bleeding. rSO_2 (regional saturation of oxygen) was monitored using the rSO_2 monitor INVOS 4100 (Somanetics Inc., Troy, MI) at the forehead (brain rSO_2) and abdomen (rectus abdominis muscle rSO_2).

28.2.4 Experimental Protocol

After establishment of stable anesthesia, animals were randomly assigned to three experimental groups in acute study, i.e. shed autologous blood (SAB) group ($n = 3$), 5 g/L recombinant human serum albumin in Saline (rHSA) group ($n = 3$), and HbV suspended in 5 % rHSA/saline solution (HbV/rHSA) group ($n = 4$). In chronic study, dogs were assigned to two groups, i.e. shed autologous blood (SAB) group ($n = 7$), and HbV suspended in 5 % rHSA/saline solution (HbV/rHSA) group ($n = 9$).

The systemic blood volume was estimated to be 86 mL per kg of the total body weight. In acute phase study, a 50 % volume of the circulation blood was withdrawn from the right femoral artery cathter at a rate of 20 ml/min. In the chronic phase study, a 40 % volume was withdrawn. Withdrawn blood was preserved in a several 50 ml syringe containing 7 ml of CPD solution (Karmi C, Kawasumi Laborataories Inc. Tokyo, Japan) in SAB group.

The hemorrhagic shock state was maintained for 1 h. Thereafter, designated isovolemic resuscitative fluid was injected intravenously. In all experiment, infusion rate of resuscitative fluid were maintained at 20 mL/kg/min. After resuscitation, no additional intravenous fluid was allowed except for the cold 5 % glucose required to measure cardiac output.

28.2.5 Measurements

In the acute phase study, 0.5 mL of arterial blood and 2.0 mL of mixed–venous blood were collected from the femoral and pulmonary arteries at the following ten time–points: before hemorrhage, immediately after hemorrhage, 1 h after the shock, immediately after resuscitation, and 0.5, 1, 1.5, 2, 3 and 4 h after resuscitation. In the chronic phase study, 10 mL of venous blood was collected from cepharic vein at the following ten time–points: before the bleeding, and 1, 3, 7, 14, 28, 56, 84, 168 and 365 days after resuscitation.

MAP was monitored through the right femoral artery, and MPAP, PCWP and CVP were through the flow directed pulmonary artery catheter connected to a transducer (5100TW, Edwards Lifesciences, Irvine, CA., USA). These transducer and EKG line were connected to a polygraph system (PEG-1000, Nihon Kohden, Tokyo, Japan). MAP, MPAP and HR were continuously monitored, and cardiac output was assessed by a thermodilution procedure with the rapid injection of cold saline (5 mL, 4 °C) in duplicate using a cardiac output measurement apparatus (Vigilance system, Edwards Critical-Care Division Irvine, CA, USA). The systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) were calculated as SVR = 79.92 × (MAP − CVP)/cardiac output, and PVR = 79.92 × (MPAP − PCWP)/cardiac output, respectively. MAP, MPAP, PCWP, CVP, cardiac output and $PtO_2(R)$ were measured at the same points stated above.

Withdrawn blood specimen (approximately 1 mL) was rapidly applied to a blood gas system (ABL555, Radio Meter Trading, Copenhagen, Denmark) to measure the pH, O_2 pressure (PaO_2) and CO_2 pressure ($PaCO_2$) of the arterial blood, and the O_2 pressure (PvO_2) and lactic acid level of the venous blood. Arterial O_2-saturation (SaO_2) and mixed venous O_2-saturation (SvO_2) were calculated by PaO_2 and PvO_2, respectively, using an O_2-equilibrium curve of the canine RBC, which was measured by a Hemox Analyzer (TCS medical products, Philadelphia, USA).

The hematocrit (Hct) was measured by using the glass capillary and centrifugation (4,500 rpm, 5 min). The Hb concentration of the arterial blood was obtained using a multi-system automatic blood cell counter (KX-21, Sysmex, Kobe, Japan). The presence of HbV in the blood interferes in the measurement of Hb concentration, thereafter the Hb concentration of the HbV/rHSA group was measured with a modified cyanomet-hemoglobin method.

The plasma Hb concentration derived from HbV was calculated as follows. Blood samples from the venous line after the HbV infusion were centrifuged at 4 °C (3,500 rpm, 10 min). The HbV molecule in the supernatant was converted to the cyanomet form using a Hemoglobin Test Wako (Wako Pure Chemical Industries, Ltd., Tokyo), and its concentration was determined by the absorption spectral measurement using a UV–vis absorption spectrophotometer (V-570, JASCO, Tokyo, Japan). The percentage of metHb within the vesicles was periodically calculated by the ratio of absorbance at 405 nm (metHb) and 430 nm

(deoxyHb) in the Soret band using a UV–vis spectrometer without destruction of the HbV (Atoji et al. 2006).

The arterial O_2-content (CaO_2 (RBC)) of the beagle dog's red blood cell (RBC) was estimated by the following equation. The oxygen delivery (DO_2 (RBC)) of the beagle dog's RBC was calculated as the product of Qt and CaO_2 (RBC).

$$CaO_2(\mathrm{RBC}) = [\mathrm{Hb}]_{\mathrm{RBC}} \times 1.34 \times \left\{SaO_2 \times 10^{-2}\right\}$$

$$DO_2(\mathrm{RBC}) = CaO_2(\mathrm{RBC}) \times 10 \times \mathrm{Qt}$$

The arterial O_2-content (CaO_2 (HbV)) of HbV was estimated by the following equation. The oxygen delivery (DO_2 (HbV)) of HbV was calculated as the product of the cardiac output (Qt) and CaO_2 (HbV).

$$\begin{aligned} CaO_2(\mathrm{HbV}) &= [\mathrm{Hb}] \times 1.34 \times \left\{1 - \text{metHb percentage} \times 10^{-2}\right\} \\ &\quad \times \left\{SaO_2(\mathrm{HbV}) \times 10^{-2}\right\} \\ DO_2(\mathrm{HbV}) &= CaO_2(\mathrm{HbV}) \times 10 \times \mathrm{Qt} \end{aligned}$$

The arterial O_2-content (CaO_2 (DO)) of the dissolved oxygen was estimated by the following equation. The oxygen delivery (DO_2 (DO)) of the dissolved oxygen was calculated as the product of Qt and CaO_2 (DO).

$$CaO_2(\mathrm{DO}) = 0.003 \times PaO_2$$

$$DO_2(\mathrm{DO}) = CaO_2(\mathrm{DO}) \times 10 \times \mathrm{Qt}$$

The total oxygen delivery (DO_2) was calculated by the following equation.

$$DO_2 = DO_2(\mathrm{RBC}) + DO_2(\mathrm{HbV}) + DO_2(\mathrm{DO})$$

The mixed venous O_2-content (CvO_2 (RBC)) of the beagle dog's red blood cell (RBC) was estimated by the following equation. The oxygen consumption (VO_2 (RBC)) of the beagle dog's RBC was calculated as the product of Qt and the difference between CaO_2 (RBC) and CvO_2 (RBC).

$$\begin{aligned} CvO_2(\mathrm{RBC}) &= [\mathrm{Hb}]_{\mathrm{RBC}} \times 1.34 \times \left\{SvO_2(\mathrm{RBC}) \times 10^{-2}\right\} \\ VO_2(\mathrm{RBC}) &= \left\{CaO_2(\mathrm{RBC}) - CvO_2(\mathrm{RBC})\right\} \times 10 \times \mathrm{Qt} \end{aligned}$$

The mixed venous O_2-content (CvO_2 (HbV)) of HbV was estimated by the following equation. The oxygen consumption (VO_2 (HbV)) of HbV was calculated as the product of Qt and the difference between CaO_2 (HbV) and CvO_2 (HbV).

$$\begin{aligned} CvO_2(\mathrm{HbV}) &= [\mathrm{Hb}] \times 1.34 \times \left\{1 - \text{metHb percentage} \times 10^{-2}\right\} \\ &\quad \times \left\{SvO_2(\mathrm{HbV}) \times 10^{-2}\right\} \\ VO_2(\mathrm{HbV}) &= \left\{CaO_2(\mathrm{HbV}) - CvO_2(\mathrm{HbV})\right\} \times 10 \times \mathrm{Qt} \end{aligned}$$

The mixed venous O_2-content (CVO_2 (DO)) of the dissolved oxygen was estimated by the following equation. The oxygen consumption (VO_2 (DO)) of the dissolved oxygen was calculated as the product of Qt and the difference between Cao_2 (DO) and CVO_2 (DO).

$$CvO_2(DO) = 0.003 \times PvO_2$$
$$VO_2(DO) = \{CaO_2(DO) - CvO_2(DO)\} \times 10 \times Qt$$

The total oxygen consumption (VO_2) was calculated by the following equation.

$$VO_2 = VO_2(RBC) + VO_2(HbV) + VO_2(DO)$$

In the chronic phase study, the collected venous blood was used for blood cell counts with an automatic blood cell counter. The rest of the blood was centrifuged (5,000 rpm, 10 min) to separate the plasma which was then ultracentrifuged (50,000 rpm, 20 min) to sediment the HbV particles from the plasma at 1, 3 and 7 days after the resuscitation with HbV/rHSA to avoid their interference by HbV particles in the plasma biochemical assays (Sakai et al. 2003). The obtained transparent serum specimens were stored at –80 °C until biochemical tests (Biken, Kyoto, Japan). The selected analyses were aspartate aminotransferase phosphatase (ALP), γ-glutamylteansferase (γ-GTP), cholinesterase (ChE), total protein (TP), albumin (ALB), creatine phosphokinase (CPK), amylase (AMY), lipase, leucine aminopeptidase (LAP), urea nitrogen (BUN), creatinine (Cre), uric acid (UA), total cholesterol (T-chol), free cholesterol (F-chol), high density lipoprotein cholesterol (HDL-chol), triglyceride (TG), free fatty acid (FFA), phospholipids, total lipids, total bilirubin (T-Bil), Fe, Cu, K, Ca, inorganic phosphate (IP), and Mg.

28.2.6 Histopahological Examination

The animals were finally euthanized with large dose of pentobarbital and exsanguination. Then autopsy was performed to get the specimen of organs (esophagus, small intestine, large intestine, liver, pancreas, spleen, thymus, lung, trachea, heart, kidney, testis, and adrenal) were obtained for a histopahological study. They were fixed in a 10 % formalin neutral buffer solution (Wako Pure Chemicals, Osaka, Japan) immediately after removal, and the paraffin sections were stained with hematoxylin & eosin (Mitsubishi Chemical Safety Institute, Kumamoto, Japan).

28.2.7 Statistical Analyses

Data are reported as mean ± standard deviation (SD) for all measurements. Data were analyzed using Stat View (SAS Institute, Inc., Cary, N.C., USA). Differences compared with the control (baseline) group were analyzed with paired *t* test, and

differences between the groups were analyzed with Mann–Whitney U test. The changes were considered significant if the p value was less than 0.05 in the acute phase study, and 0.01 in the chronic phase study.

28.3 Results

28.3.1 Acute Phase Study

Beagle dogs of all groups tolerated well the 50 % bleeding inducing hemorrhagic shock and resuscitation. They survived for 4 h after the resuscitation without any change in their appearance.

Circulation

MAP before hemorrhage was 102 ± 19 mmHg on the average; it decreased significantly to 19 ± 5 mmHg immediately after hemorrhage (Fig. 28.1). After resuscitation, both the SAB and HbV/rHSA groups showed immediate recovery and stable values for the 4 h. The rHSA group showed significantly lower MAP than the HbV/rHSA group at 1, 3, and 4 h after infusion.

All groups showed significantly higher MPAP immediately after infusion than the baseline values. However, there were not significant differences between groups. After that, all groups showed almost stable values. The HbV/rHSA group showed slightly lower PCWP after resuscitation than the other groups. The SAB group showed slightly higher CVP after infusion than the other groups.

There was no significant change in the time course of the HR during the experiment. CO before hemorrhage was 1.4 ± 0.3 L/min on the average; it decreased significantly to 0.2 ± 0.1 L/min immediately after hemorrhage. After resuscitation, all groups showed immediate recovery, and rHSA group showed significantly higher values than the baseline. The SAB group showed significantly lower values than the HbV/rHSA group at 0.5, 1, 1.5, and 3 h after resuscitation.

All groups showed significantly lower SVR at 0, 0.5, 1, 1.5, and 2 h after resuscitation than the baseline values. The rHSA group showed lower SVR than the HbV/rHSA group at 0, 0.5, 1, 1.5, 2 h after infusion. The 50 % hemorrhage increased the PVR, however, after the hemorrhage, all groups showed stable values for 4 h of the observation period.

Blood gas analysis (Fig. 28.2).

The pH value decreased to 7.13–7.22 after hemorrhage, but both the SAB and HbV/rHSA groups showed immediate recovery and stable values for the 4 h. The rHSA group tended to recover late. BE decreased to 11.5–15.6 mmol/L after hemorrhage, but all groups showed gradual recovery to the initial level after infusion. The lactic acid decreased to 3.07–5.07 mmol/L after hemorrhage, but all groups showed gradual recovery like BE. As a result of the hyperventilation, the slight elevation of PaO_2 and the decline of $PaCO_2$ were seen after hemorrhage. However, all groups showed recovery and similar tendency. PvO_2 before

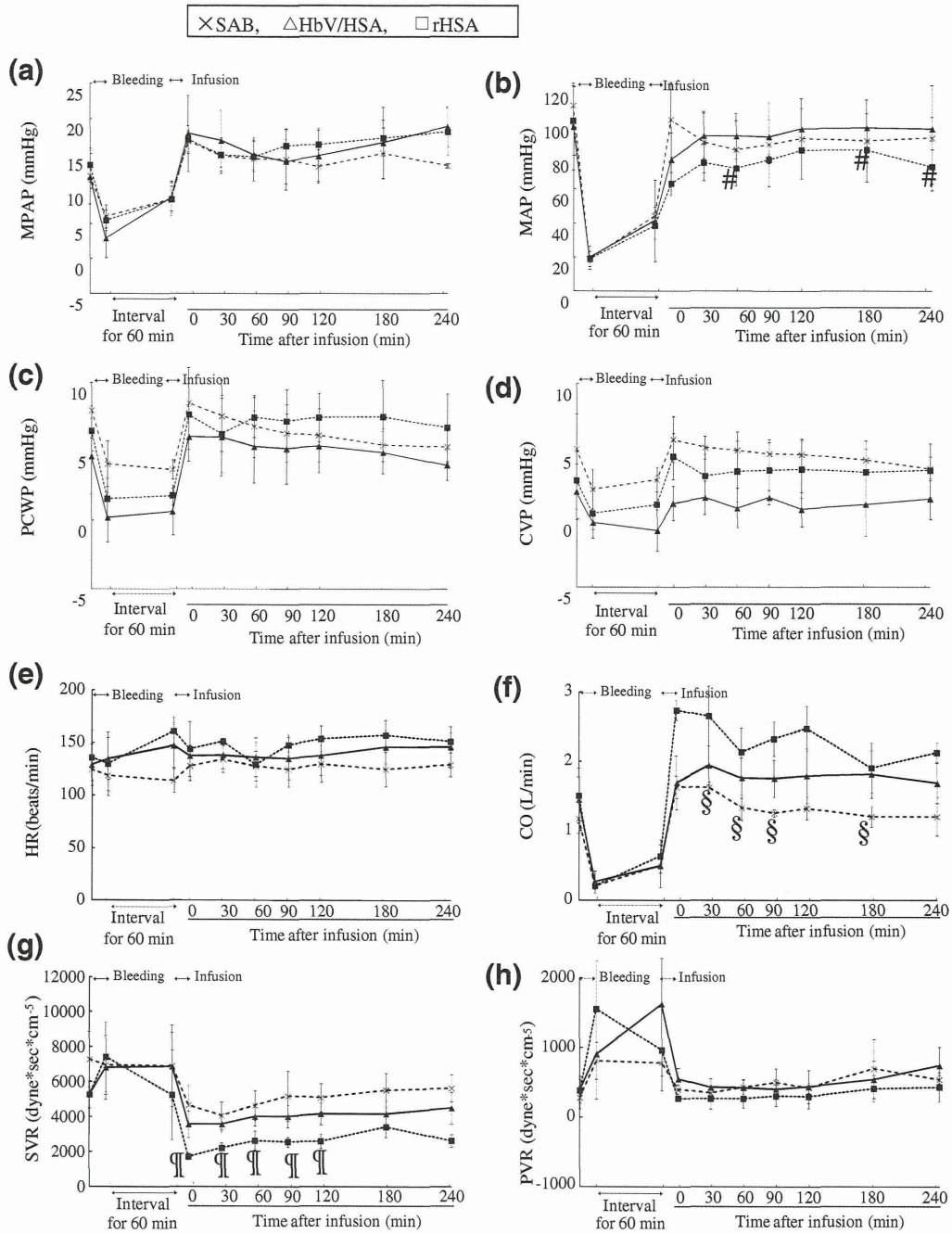

Fig. 28.1 Changes in mean pulmonary arterial pressure (MPAP), mean arterial pressure (MAP), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), heart rate (HR), cardiac output (CO), systemic vascular resistance (SVR), and pulmonary vascular resistance (PVR) during hemorrhagic shock and resuscitation with infusion of rHSA alone, shed SAB and HbV/rHSA. The values are mean ± SD. #,§: significantly different between HbV/rHSA group ($p < 0.05$)

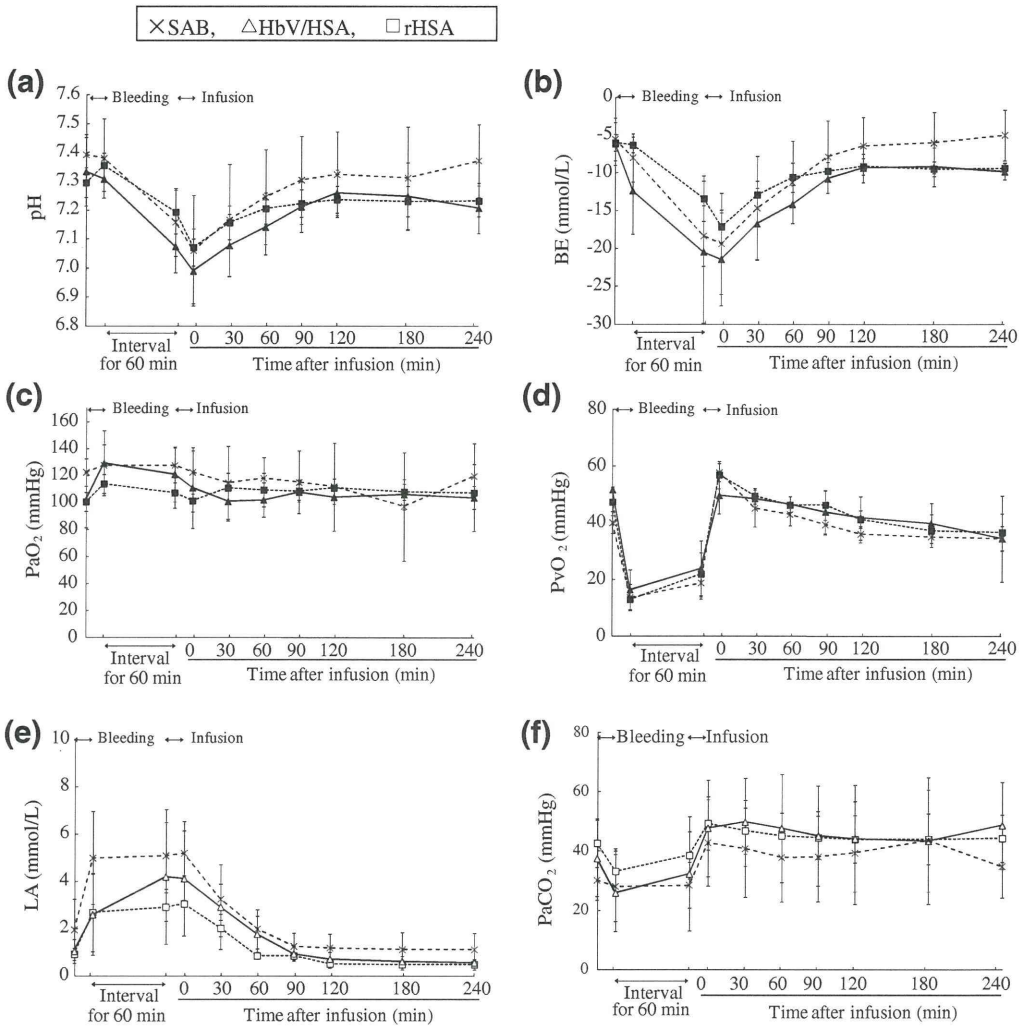

Fig. 28.2 Changes in pH, PaO_2, $PaCO_2$, base excess (BE), lactate, and PvO_2 during hemorrhagic shock and resuscitation with infusion of rHSA alone, SAB and HbV/rHSA. The values are mean ± SD. There was no significant difference between rHSA or SAB and HbV/rHSA group

hemorrhage was 48 ± 5 Torr on the average; it decreased significantly to 15 ± 6 Torr immediately after hemorrhage. After resuscitation, all groups showed immediate recovery and similar tendency.

Hematology (Fig. 28.3).

There was no significant change after bleeding in Hct for all groups. As a result of the dilution of blood, the rHSA and HbV/rHSA groups showed significantly lower values than the baseline values, while the SAB group showed higher values during the experiment (Fig. 28.3c). In the HbV/rHSA group the total Hb levels before hemorrhage was 10.4 ± 1.6 g/dL, and after resuscitation it was 13.8 ± 1.6 g/dL at 0 h, and 9.9 ± 1.2 g/dL at 4 h (Fig. 28.3b). The concentration of Hb derived from HbV was 4.2 ± 0.5 g/dL (37.5 ± 4.5 % of total Hb) at 0 h,

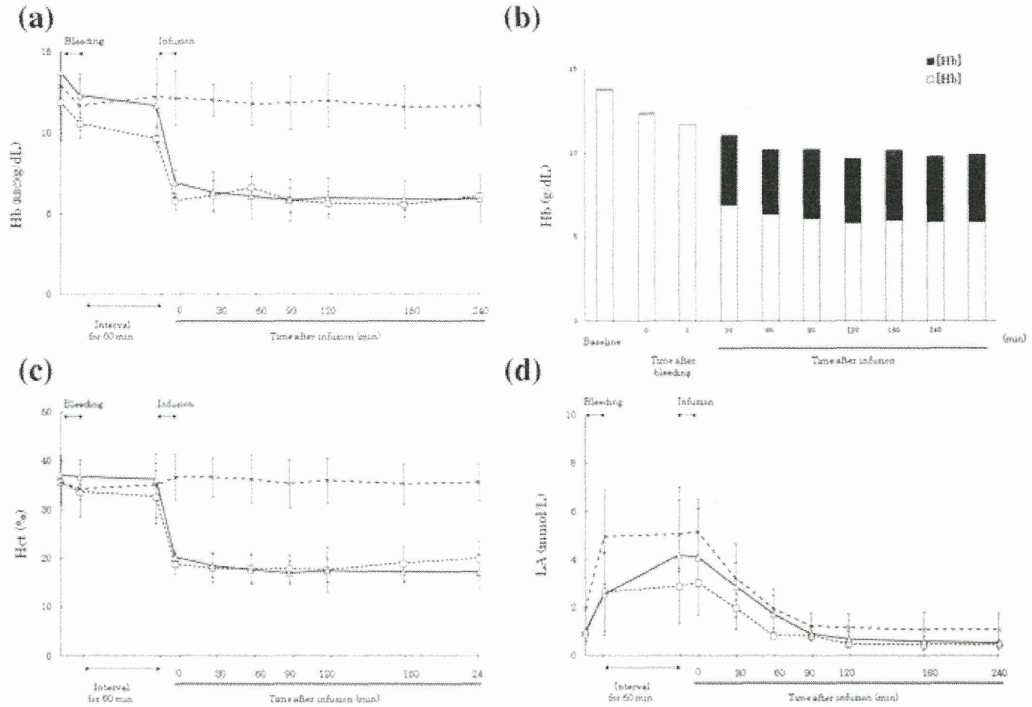

Fig. 28.3 Change in hemoglobin (Hb) concentration, hematocrit (Hct), and lactic acid (LA) level during hemorrhagic shock and resuscitation with infusion of rHSA alone, SAB, and HbV/rHSA. The values are mean ± SD. Composition of Hb concentration in the whole blood in HbV/rHSA group (B)

and 4.0 ± 0.8 g/dL (40.5 ± 8.1 % of total Hb) at 4 h. The level of metHbV increased to 9.1 ± 3.0 % at 4 h.

Oxygen delivery and consumption (Fig. 28.4).

As regards to the oxygen delivery and consumption, the rHSA group tended to show lower DO_2 than the other groups after the resuscitation, and showed significantly lower value than HbV/rHSA group 3 h after resuscitation (Fig. 28.4a). In the HbV/rHSA group, DO_2(HbV) was 34–38 % of the total DO_2. Oxygen consumption was not significantly different between HbV/rHSA group and SAB, or rHSA group. In HbV/rHSA group VO_2(HbV) showed 26–29 % of the total VO_2.

28.3.2 Long Term Study

Beagle dogs of all groups tolerated well the 40 % bleeding inducing hemorrhagic shock and resuscitation. They survived without any change in their appearance until their intentional sacrifice. The body weight of beagle dogs before resuscitation was 7.2 ± 0.3 kg, which increased monotonously to 12 ± 1.8 kg at one year after resuscitation in both groups (Fig. 28.5). The Hct before the resuscitation

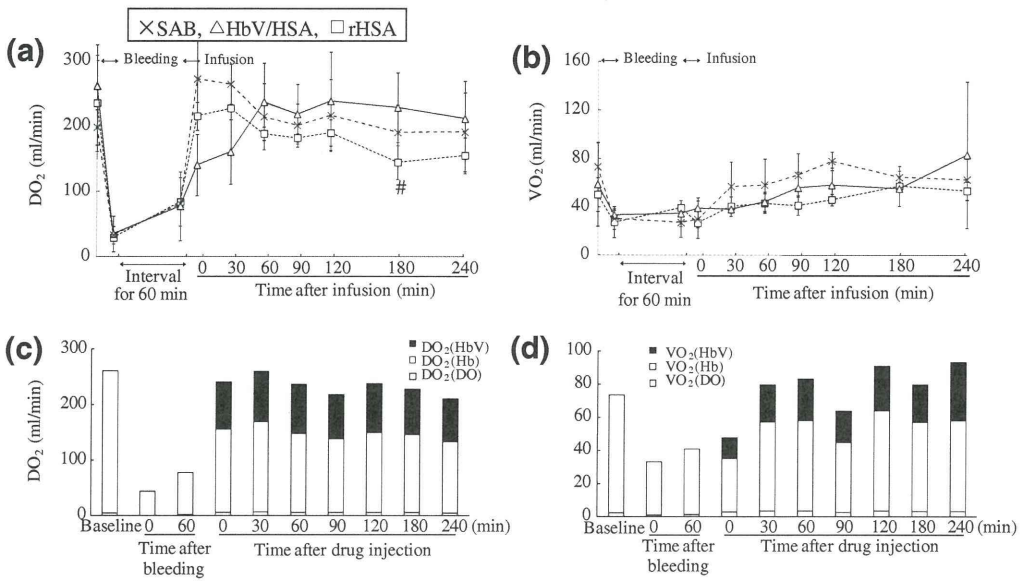

Fig. 28.4 Changes in oxygen delivery (DO_2), and oxygen consumption (VO_2) during hemorrhagic shock and resuscitation with infusion of rHSA alone, sSAB and HbV/rHSA (*Top*). The rates of oxygen delivery derived from dissolved oxygen in plasma (DO_2(Plasma)), hemoglobin of RBCs (DO_2(RBC)), and HbV (DO_2(HbV)) in total oxygen delivery (DO_2) of the HbV/rHSA group and oxygen consumption derived from dissolved oxygen in plasma (VO_2(Plasma)), hemoglobin of RBCs (VO_2(RBC)), and HbV (VO_2(HbV)) in total oxygen consumption (VO_2) of the HbV/rHSA group (*Bottom*). The values are mean ± SD. # significantly different versus the HbV/rHSA group ($p < 0.05$)

was approximately 35 %. It decreased to about 29 % for the HbV/rHSA group after recovery from acute phase study. However, it showed monotonic increase; at 7 days, the Hct showed a complete recovery to the baseline level (about 35 %). Although Ht recovered consistently in HbV/rHSA group, we could see distinguished significances until 2 months. White blood cell and platelet counts showed non-significant changes between the HbV/rHSA and SAB groups, and then maintained rather steady values.

Regarding the plasma biochemical tests, AST and ALT showed increases on Day 1 in both HbV/rHSA and SAB groups, but it reverted to the original level on Day 3 (Fig. 28.5). LDH showed decreases on Day 1 in both groups, but both groups showed gradual increases until Day 7. The HbV/rHSA group tended to show lower ALP than the SAB group, and showed significantly lower values than baseline after 168 and 365 days. γ-GTP, ChE, TP, and ALB showed stable values for 1 year. CPK showed increases on Day1 in both groups, but it reverted to the original level on Day 3. Amylase showed non-significant change between HbV/rHSA and SAB group for 1 year. Lipase showed significant decrease in HbV/rHSA group on Day 7, but it tended to show gradual recovery. LAP, BUN, Cre, and UA showed non-significant changes between HbV/rHSA and SAB group. Regarding plasma lipid components in the HbV/rHSA group, Total-cholesterol level and Free-cholesterol level showed significant increases on Day 7. However,

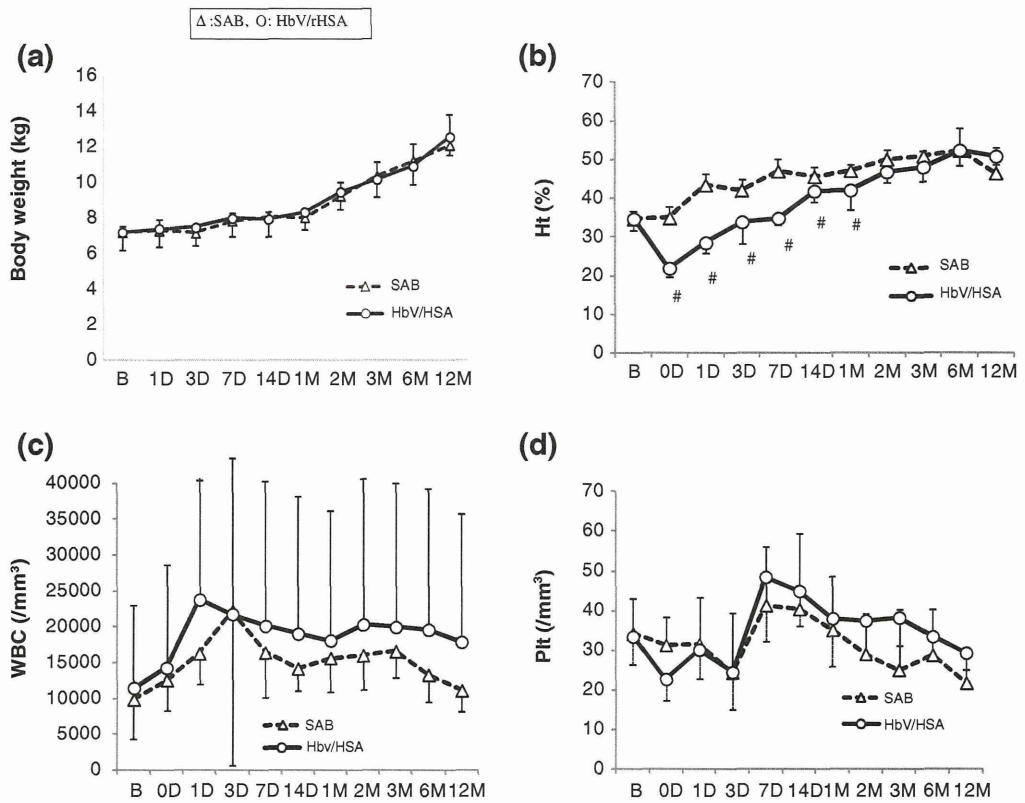

Fig. 28.5 One-year observation of changes in body weight, hematocrit (Hct), white blood cells count (WBC), and platelet count (PLT) after resuscitation with infusion of SAB and HbV/rHSA. The values are mean ± SD. # significantly different versus the autologous shed blood group ($p < 0.01$)

they returned to their original levels at Day 14 (Fig. 28.6). HDL-Chol (High Density Lipoprotein-cholesterol) showed significant decrease for HbV/rHSA group on Day 1, but it reverted to the original level on Day 3. Trigriseride (TG) and Free Fatty Acid (FFA) showed non-significant change between the HbV/rHSA and SAB groups. Phospholipids and Total Lipid showed significant increases in the HbV/rHSA group on Day 7, but they showed non-significant change after Day 3. Total bilirubin (T-Bil) and Fe maintained steady values. Copper ion showed significant increase in the SAB group on Day 1, but it showed similar tendency after Day 3. K, Ca, IP, and Mg showed stable values for 1 year.

28.3.3 Histopathological Study

In acute phase study, sinusoid of the liver showed the eosinophilic fine granular material in the HbV/rHSA group (Fig. 28.7). The red pulp zone of the spleen showed eosinophilic fine granular material in the HbV/rHSA group. These findings