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Cross-linked inulin as a potential plasma expander: Biochemical properties and physiological characterization in a rabbit model

Suping Li[a,b,1], Tao Hu[a,1], Yali Chen[a], Chunyang Zheng[a], Tao Liu[a,b], Guanghui Ma[a], Zhiguo Su[a,*]

[a] National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, PR China
[b] Graduate School, Chinese Academy of Sciences, Beijing 100190, PR China

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ABSTRACT

Inulin, a plant-source polysaccharide, was cross-linked and evaluated as a new potential plasma expander *in vitro* and *in vivo*. The cross-linked inulin (CLI) in three different molecular sizes (54, 100 and 146 kDa) was characterized by gel filtration chromatography coupled with multi-angle laser light scattering. Their *in vivo* physiological properties, including blood pressure and organ function, were tested in a rabbit model. Compared to the other two CLI samples, the CLI2 with a molecular weight (M_w) of 100 kDa is advisable as a potential plasma expander for its strong expansion efficacy and no organ dysfunction. Moreover, CLI2 showed a high colloid osmotic pressure (COP) where the COP of 3% CLI2 is equal to that of 6% HES. Thus, much less CLI2 can be infused with the same effects as normal HES, and its potential side effects can be minimized.

1. Introduction

Natural polymers have received much attention for their biocompatibility, bioactivity and biodegradability (Steinbuchel & Marchessault, 2005). Modern plasma expanders (pullulan, dextran or hydroxyethyl starch) consist of water-soluble polysaccharides that are effective in the treatment of intravasal volume deficiency. HES is a highly branched amylopectin with ether-linked hydroxyethyl groups and has been extensively studied as a plasma expander. However, HES treated-patients suffer from allergic reactions, bleeding defects and platelet damage (Frese et al., 2000; Messmer, 1987; Schortgen et al., 2001). The side effects of HES depend on the molecular weight (M_w) and its degree of substitutes (DS) (Treib, Baron, Grauer, & Strauss, 1999). For example, HES 450 (M_w = 450 kDa) may result in bleeding complications and pursuits (Yoshida, Amino, & Kishikawa, 1984), whereas HES 70 (M_w = 70 kDa) showed a less efficacy of volume expansion due to its rapid renal elimination (Treib et al., 1999). Accordingly, HES 130 (M_w = 130 kDa) is more effective for volume replacement therapy and has fewer side effects due to its M_w (Sudhakar & Lakshmi, 2008).

In addition, the effects of polysaccharide (e.g., dextran, DEX) on blood are related to M_w. The rheologic effect of DEX 40 (M_w = 40 kDa) solution is especially pronounced since this solution can reduce viscosity of whole blood more with the corresponding degree of hemodilution compared to DEX 60 (M_w = 60 kDa) and DEX 70 (M_w = 70 kDa) (Dewachter, Laxenaire, Donner, Kurtz, & Stoltz, 1992). After dilution with various colloid plasma expanders, the low shear-rate viscosity is reduced by DEX 40 and increased by DEX 60 and DEX 70. Unlike DEX 60 and 70, DEX 40 solutions have a beneficial effect on red cell rouleau formation since they increase the time of red cell aggregation. A new finding regarding the beneficial rheologic effects of DEX showed that leukocyte adherence may be involved (Steinbauer, Harris, Leiderer, Abels, & Messmer, 1998).

The aim of this work is to develop a new plasma expander with a proper M_w that can expand the blood volume. Moreover, its products would produce fewer side effects than other plasma expanders. In the present work, inulin is chosen as the backbone polymer for its prior history of intravenous use in humans, where it is served as a diagnostic agent for the measurement of glomerular filtration rate (van Rossum et al., 2005).

Inulin, a plant-source polysaccharide from *Cichorium intybus* (chicory), *Dahlia pinuata Ca*V. (dahlia) and *Helianthus tuberosus* (Jerusalem artichoke), is a polydisperse polysaccharide consisting mainly, if not exclusively, of β (2-1)-fructosyl fructose units with normally, but not necessarily, one glucopyranose unit at the reducing end (GFn) (Suzuki, 1993). Inulin, with its non-toxic and biocompatible characteristics (Wu & Lee, 2000), promises a wide application in medicine, indicating that inulin has potential as a plasma expander. However, inulin may induce rapid renal excretion because of its low M_w. Cross-linkage of inulin to enhance its M_w is a potential strategy to circumvent this disadvantage. There-

* Corresponding author. Tel.: +86 10 62561817; fax: +86 10 62561813.
E-mail address: zgsu@home.ipe.ac.cn (Z. Su).
[1] Both authors contributed equally to the work.

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fore, cross-linked inulin (CLI) with a proper M_w is important for its application as a plasma expander.

The present study was aimed at establishing a CLI with a proper M_w as a plasma expander. Accordingly, we prepared three CLI samples with different M_w using epichlorohydrin (EP) as a bifunctional cross-linker to conjoin inulin molecules. The molecular structure, dispersity and M_w of the three CLI samples were measured and characterized. Their *in vitro* physiological properties were analyzed, including colloidal osmotic pressure (COP), effect on red blood cells and viscosity. Their *in vivo* physiological properties including blood pressure and organ function were tested in an ameliorative hemorrhagic shock rabbit model. These properties were compared with those of three commercial plasma expanders (HSA, HES and NS) to establish the potency of CLI as a plasma substitute.

2. Materials and methods

2.1. Preparation of CLI with different M_w

A mixture of 2.3 g inulin (average M_w of 3.2 kDa, Orifti, Belgium) in 10 ml of 20% (w/w) NaOH solution was mechanically stirred in a flask (50 ml) overnight at 30 °C. The mixture was then heated to 35 °C, followed by the rapid addition of 2 ml EP and constant stirring at a rate of 180 rpm. The reaction equation is as following:

$$\text{inulin} - \text{OH} + \text{EP} \xrightarrow{\text{NaOH}} \text{inulin} - \text{CH}_2\text{OCH}_2\text{CHOHCH}_2\text{OCH}_2 - \text{inulin} \quad (1)$$

The reaction was stopped after 4 h by the addition of acetone, followed by the removal of acetone using a separating funnel. The pH of the aqueous solution was decreased to 12 by the addition of 6 mol l^{-1} HCl. The solution was kept overnight at 45 °C. After cooling, the solution was further neutralized with 6 mol l^{-1} HCl and was precipitated with the addition of ethanol. The cross-linked inulin concentration was adjusted to 0.3 g ml^{-1} by the addition of distilled water and then was ultrafiltered with different cut-off membrane under pressure (2 bars) according to the object M_w. Finally, the solution was lyophilized.

2.2. Determination of physical parameters

CLI was characterized by gel filtration chromatography coupled with multi-angle laser light scattering (GFC–MALLS). The GFC–MALLS was performed using an Agilent 1100 HPLC with a TSK5000 column (7.5 mm × 300 mm i.d., Tosoh Bioscience LLC, Japan). The column was eluted with 50 mmol l^{-1} sodium phosphate containing 0.15 mol l^{-1} sodium sulfate (pH 7.4) at a flow rate of 0.5 ml min^{-1}. The detector consists of a DAWN EOS laser photometer (Wyatt Technology, Santa Barbara, CA) equipped with a 690 nm laser, an Optilab DSP differential interferometer refractometer (Wyatt Technology, Santa Barbara, CA), quasi-elastic light scattering (QELS, Wyatt Technology, Santa Barbara, CA) and a diode array detector (Agilent Technologies, Mountainview, CA).

2.3. Determination of colloidal osmotic pressure (COP)

The COP was analyzed using a membrane colloid oncometer with a 20 kDa semipermeable membrane (BMT 923, Delta-Pharma, Pfullingen, Germany). The COP of the samples (~150 μl) was measured three times at 22–24 °C.

COP has been produced using the chemical potential change of solvent in the presence of solute. The osmotic force to balance the chemical potential can be calculated by the following equation (He, Chen, & Dong, 1990):

$$\frac{\pi}{c} = RT\left(\frac{1}{M_{cop} + A_2c + A_3c^2 + \cdots}\right) \quad (2)$$

where π is oncotic pressure, c is solute concentration, R is the gas constant, T is temperature in degrees, $1/M_{cop}$ is the first virial coefficient, A_2 is the second virial coefficient that measures the solution ideality. Eq. (1) can be rearranged into a linear form by ignoring the higher-order virial coefficient term when A_2 is low:

$$\frac{\pi}{c} = \frac{RT}{M_{cop}} + RTA_2c \quad (3)$$

M_{cop} can be calculated by $(\pi/c)_{c=0}$, which is the intercept obtained from the linear least-squares regression of π/c versus c. Values for the second virial coefficient (A_2) were calculated from the slope of π/c versus c using linear least-squares regression.

3. Animals and protocols

3.1. Experimental animals

New Zealand white rabbits (male, 2.3–2.8 kg) were obtained from the Animal Center of Peking University Health Science Center. They were maintained at 24 ± 1 °C and at a relative humidity of 50 ± 1%. Animals were fasted for 24 h with water ad libitum before the experiment.

3.2. Randomization and study groups

Animals were randomly allocated to six groups according to the fluid used for replacement of blood loss:

CLI1 group: (2.7% CLI1 with M_w of 54 kDa in NS solution)
CLI2 group: (3% CLI2 with M_w of 100 kDa in NS solution)
CLI3 group: (4.8% CLI3 with M_w of 146 kDa in NS solution)
NS group: (0.9% NaCl, Zizhu Pharmaceutical, Beijing, China)
HES group: (6%, Voluven 130/0.4, Fresenius, Beijing, China)
HSA group: (5% human serum albumin, Hualan Biotechnology Inc., China)
Six rabbits were used for each volume expander.

3.3. Surgical procedures

Animals were placed supinely on the operating table. Anesthesia was induced by ear intravenous injection of pentobarbital at 0.045 ml kg^{-1}. The right jugular vein and right carotid artery were surgically exposed. Then, an arterial catheter with a Y-tube and venous catheter filled with a heparin (30 IU/ml) were inserted via the right carotid artery and right jugular vein. The right jugular vein was used for the administration of fluids. One lead of the artery catheter was linked to a BL-420E Biomedical Recorder (Chengdu TME Technology Co. Ltd., Chengdu, China) to determine the blood pressure and heart rate. Another one was used to extract blood. After a 20 min stabilization period, the first set of measurements (baseline) was performed. Then, a blood loss of 15 ml kg^{-1} of blood (30% of blood volume) was started at 1 ml kg^{-1} min^{-1} to keep the MAP between 40 and 50 mmHg. After 10 min of the hemorrhagic period, the six experimental solutions were infused. In each experiment, the quantity of solution was equal to the volume of blood lost to induce the hemorrhage. The signal was recorded for about 2 h using a BL-420E Biomedical Recorder. The animals were reared on laboratory feed and injected with penicillin for 7 days.

3.4. Determination of the in vitro blood properties

Heparin-supplemented rabbit blood was mixed with the same volume of each plasma expander. The viscosity was measured in a capillary viscometer (Model LG-R-80B, Steellex Co., China) at 37 °C with shear rates varying from 1 to 200 s^{-1}. EDI and EAI were determined by laser diffraction analysis using an ektacytometer (Model

LG-B-190R, Steellex Co., China) as described elsewhere (Yao et al., 2001). All the measurements were carried out at 37 °C.

3.5. Biopsy

After 14 days, animals were sacrificed and organs, including kidney, liver, lung, spleen, heart, brain, were dissected out and cleaned with saline. Then, these organs were fixed for 24 h by immersion in 10% formalin solution. Paraffin sections of the organs (4 μm thick) were prepared for hematoxylin and eosin (H&E) staining, followed by examination using a BI-2000 medical image analysis system (Chengdu TME Technology Co. Ltd., Chengdu, China).

3.6. Statistical analysis

Results were expressed as means ± S.D. Statistical significance was tested using Student's unpaired *t*-test to compare two groups. Changes in variables from baseline or shock during volume replacement therapy were assessed in each group using Student's paired *t*-test. The value of $p < 0.05$ and $p < 0.01$ were considered to indicate significant and highly significant differences between the groups, respectively.

4. Results

4.1. Physics of CLI with different M_w

Physical parameters of the colloid solutions, including molecular mass, hydrodynamic radius, dispersity, A_2 and colloid osmotic pressure, were determined by SEC–MALLS and membrane osmometry (Table 1, Figs. 1 and 2).

Colloid particles were analyzed by SEC–MALLS and the molecular mass of the colloids were thus calculated by Astra Software 4.90. As shown in Fig. 1A, CLI3 was the earliest eluted sample, followed by CLI2, HES130, HSA and CLI1. This indicated that hydrodynamic volume and M_w of CLI3 is the largest of the five samples. These results are in agreement with the values calculated by Astra Software 4.90. However, the M_w of CLI2 is close to that of HES, although the R_h of CLI2 is larger than that of HES, presumably due to the exceptionally high A_2 of CLI2, which is related with the solvent accessible surface area (Vandegriff, Michael, Rohlfs, & Winslow, 1997). This indicated that the hydroxyls of CLI2 are more exposed to the surrounding solvent. In addition, CLI3, CLI2, CLI1 and HSA showed a narrower polydispersity than HES (Table 1), which facilitates renal metabolism regardless of its heterogeneity. These can be verified by the peak shape of colloids in Fig. 1A and the results calculated by Astra Software 4.90 (Fig. 1B and Table 1).

Fig. 2A shows the COP of the colloid solutions at different concentrations. Interestingly, the CLI solutions showed a much higher COP than other plasma substitutes. For example, the COP of 2.7% CLI1 and 3% CLI2 are higher than 5% HSA and equivalent to 6%

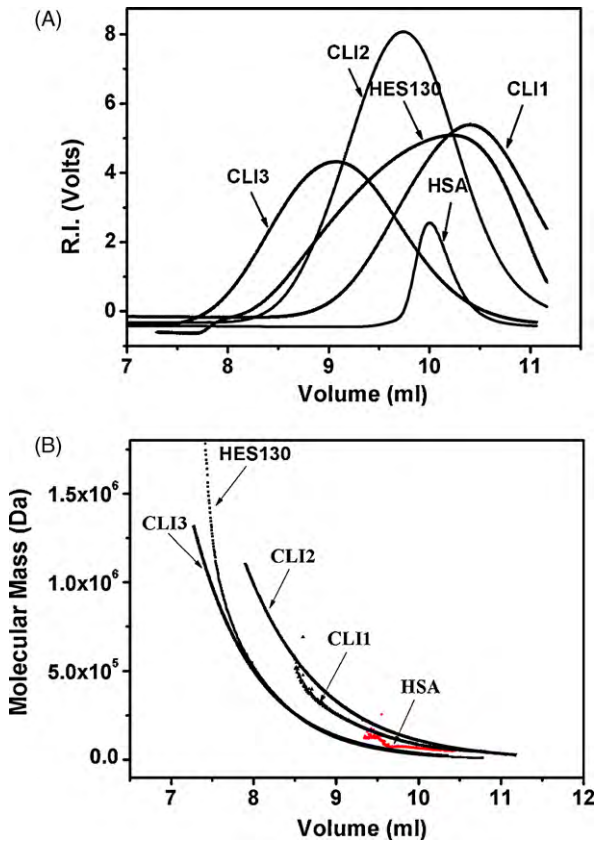

Fig. 1. Molecular mass distribution of the plasma expanders. The refractive index (R.I.) response (A) and molecular mass distribution (B) of the samples were measured using a TSK 5000 column.

HES. Thus, CLI1 and CLI2 may have volume-expanding capacity comparable to HES and HSA, but can be infused at a much lower dosage. All of the data in Fig. 2B had a good fit using linear regression. This justifies using Eq. (3) for this analysis, because higher-order virial coefficients were not necessary to describe the data. The slopes (i.e., A_2) of HSA, HES and CLI3 are close to zero, whereas the slopes of CLI1 and CLI2 were larger. This may be due to the higher number of hydroxyl groups exposed in solvent.

4.2. Biochemical and physiological properties of CLI

Fig. 3 represents the dependence of viscosity upon M_w. With the increase of M_w, the viscosity is increasing in CLI-treated group. In comparison with the viscosity of blood, NS and HES showed lower viscosity, whereas CLI3 and HSA showed higher viscosity. The vis-

Table 1
The physical parameters of the five colloidal solutions.

	HSA	HES130	CLI1	CLI2	CLI3
M_n (kDa)[a]	67.6	54.8	3.9	62.3	100.2
M_w (kDa)[b]	67.7	129.4	5.4	98.1	146.1
Polydispersity	1.00	2.36	1.37	1.58	1.46
Hydrodynamic radius (nm)[c]	3.5	4.9	3.1	6.3	8.2
COP (mmHg)[d]	18.9 ± 1.3	29.7 ± 1.6	30.1 ± 1.3	29.5 ± 1.4	28.9 ± 1.8
A_2 (10^{-3} cm^3 mol g^{-2})[e]	2.06	2.89	13.35	15.62	3.05
Concentration (g dl^{-1})	5.0	6.0	2.7	3.0	4.8

[a] The number average molecular weight.
[b] The weight average molecular weight.
[c] Hydrodynamic radius.
[d] Colloid oncotic pressure as determined by membrane osmometry.
[e] The second virial coefficient.

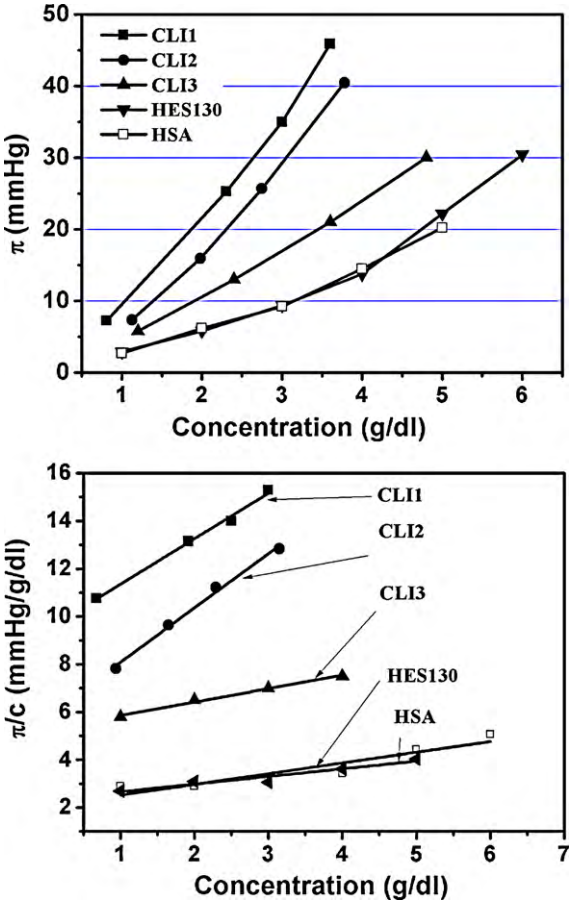

Fig. 2. Colloid osmotic pressure (COP) of the plasma expanders. The COP (A) and the COP data plotted as π/C versus C (B) were measured as a function of concentration.

cosities of CLI1 and CLI2 (33 and 36 mPa s^{-1}, respectively) were close to that of the blood (32.5 mPa s^{-1}), were higher than NS and HES (25.0 and 26.0 mPa s^{-1}, respectively) and were lower than CLI3 and HSA (39 and 46 mPa s^{-1}, respectively).

Normal red blood cells can pass through capillary vessels by deformation. This deformation ability, as reflected by EDI, can regulate blood viscosity and microcirculation. As shown in Fig. 4, as compared to the NS-treated group, the *in vitro* EDI in all the other groups were improved except for CLI1 groups ($p < 0.05$). Moreover, the EDI values for the CLI groups are a function of their M_w.

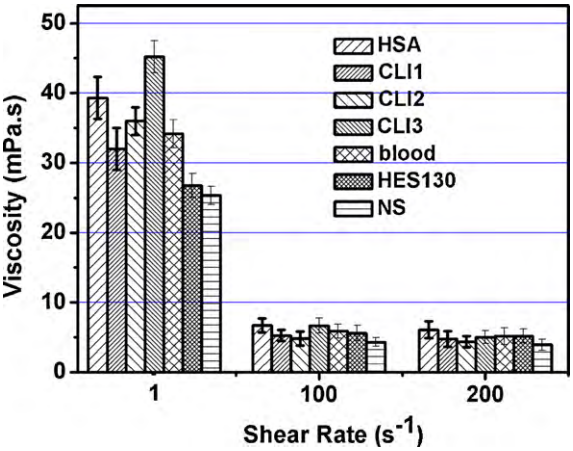

Fig. 3. The *in vitro* viscosity of the six therapy groups and blood.

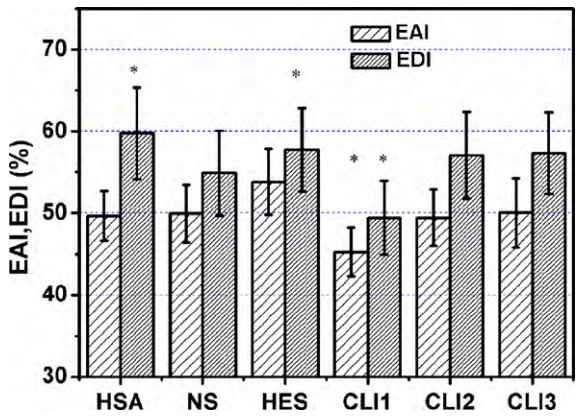

Fig. 4. The *in vitro* EDI and EAI of the different plasma expanders. $^{*}p < 0.05$ versus the NS group.

EAI is the ability of erythrocyte to aggregate in blood or in the formation of reticulation structure with macromolecules in plasma. Aggregated erythrocytes cannot deliver oxygen and could increase blood viscosity. The NS-treated group shows a lower EAI than the HES-treated group and higher EAI than the HSA-treated group (Fig. 4). In the CLI-treated group, the EAI values are related to their M_w. The CLI1-treated group had a lower EAI value than the NS-treated group, whereas CLI2- and CLI3-treated groups had EAI values close to the NS-treated group.

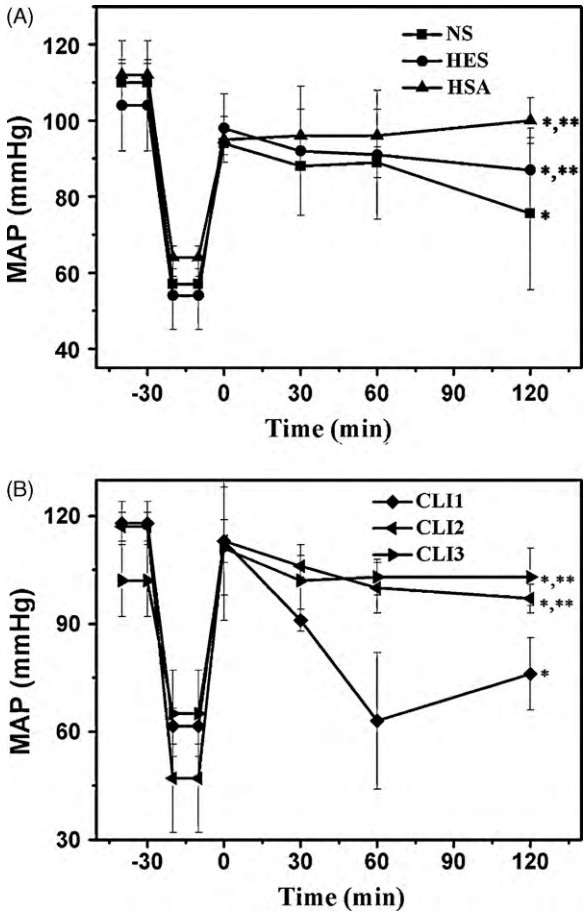

Fig. 5. The MAP of the six therapy groups. $^{*}p < 0.01$ versus shock, $^{**}p < 0.05$ versus NS.

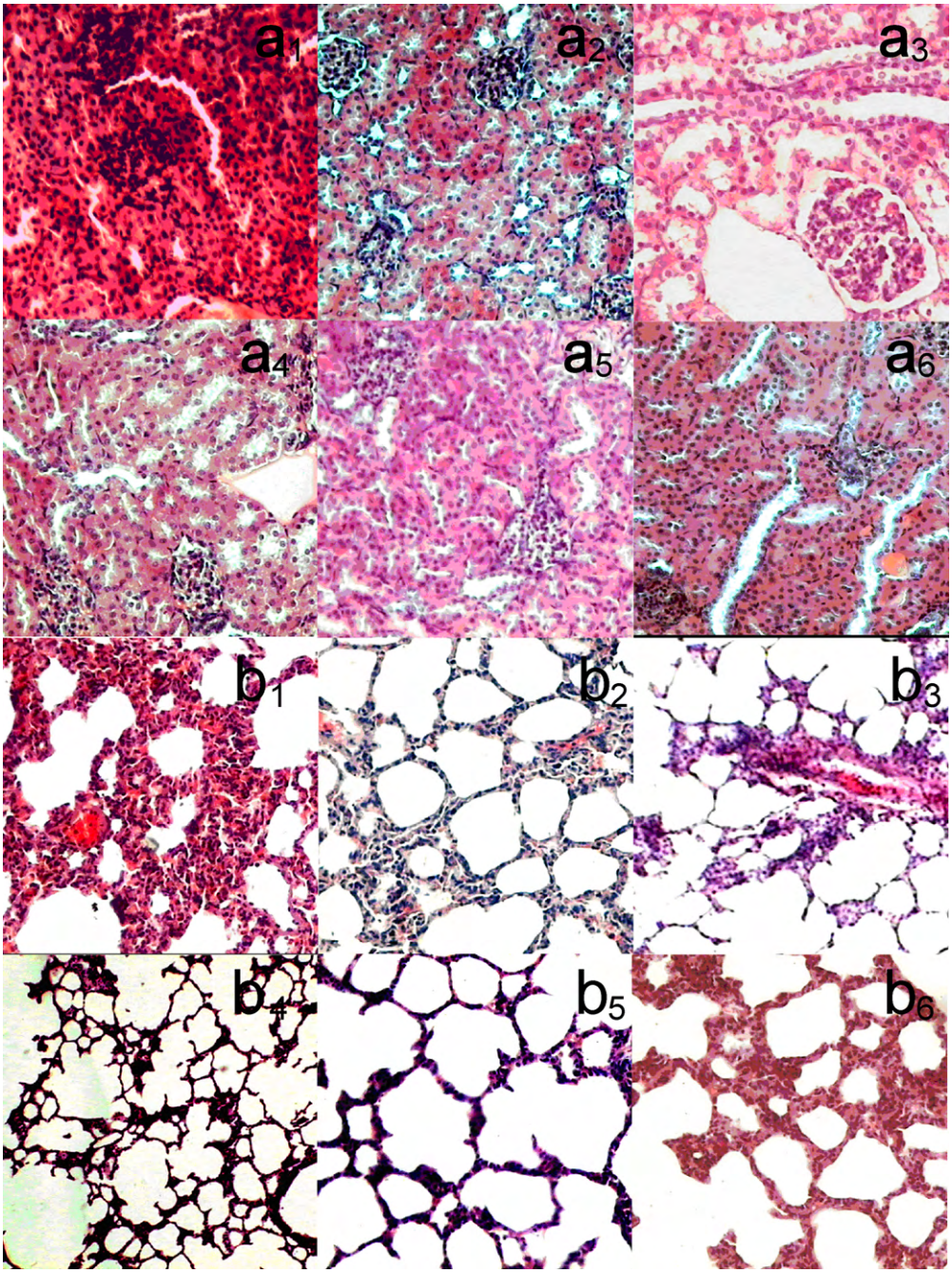

Fig. 6. The biopsy analysis of the organs of the six therapy groups. Paraffin sections of kidney (A, 400×) and lung (B, 400×) were prepared for H&E staining. 1. NS group; 2. HES group; 3. HSA group; 4. CLI1 group; 5. CLI2 group; 6. CLI3 group.

4.3. In vivo hemodynamic characteristics

Mean artery pressure (MAP) is an important hemodynamic variable that is affected by blood volume. As shown in Fig. 5, the MAP was lowered to approximate 50 mmHg during blood removal in all the therapy groups at a similar hypotension level. During the resuscitation phase, MAP progressively improved for all therapy groups. However, as compared to the HSA- and HES-treated groups, CLI2 and CLI3 can provide a more rapid restoration of MAP (Fig. 5). NS and CLI1 showed a relatively slow restoration of MAP that decreased rapidly after 2 h of transfusion.

4.4. Effect of transfusion on biopsy specimens

As revealed by the biopsy analysis (Fig. 6), the kidney and the lung were seriously damaged in the NS-treated group. The edema of the renal collecting ducts and thickening of the connective tissue proliferation with congestion were observed, because NS cannot effectively expand the blood volume. Presumably, the crystalloid property of NS solution may favor fluid shifts from the intravascular compartment to the interstitial compartment, thereby increasing the risk of edema formation. In contrast, there are a small number of foreign bodies in the kidneys in the CLI3-treated group, presumably because the CLI3 cannot be metabolized smoothly due to its

larger M_w. In addition, edema of lungs was found in the CLI3-treated group, presumably because the higher viscosity hindered blood flow. In the other groups, glomerular structure was obvious, and necrosis and edema of renal collecting ducts were not observed.

5. Discussion

The ideal plasma substitute for clinical use should: (i) maintain the proper COP in blood over a long period of time; (ii) be free of side effects such as allergic reactions or organ dysfunction; (iii) be inexpensive and with a long shelf life. Therefore, the CLI in three different molecular sizes (54, 100 and 146 kDa) was prepared in the present study and evaluated as potential artificial colloidal solutions *in vitro* and *in vivo*. The principle finding of this study is that the physiochemistry of CLI (viscosity, effect on red blood cells and COP) is dependent on M_w *in vitro,* and CLI2 with a M_w of 100 kDa is a potential plasma expander. In addition, COP of 3% CLI2 is equal to that of 6% HES and 5% HSA that can lead to a rapid restoration of blood pressure with infusion at a much lower dosage. This agreed well with the MAP results *in vivo*. Moreover, CLI2 can be renally metabolized and showed no organ dysfunction by biopsy analysis, due to its proper M_w and lower dispersity.

Viscosity is an important factor for putative plasma expanders, because the plasma viscosity is strongly correlated with M_w (Feng et al., 2006). It has been proposed that the viscous drag exerted on the endothelial cells by flowing blood, induces wall shear stress and triggers flow-induced dilation (Sakai, Yuasa, Onuma, Takeoka, & Tsuchida, 2000). Wall shear stress is expressed as $8\eta V_m/D$, where V_m is the mean red blood cell velocity, η is the viscosity, and D is the vessel diameter. Transfusion with a commercial plasma expander (e.g., HES) reduced blood viscosity and lowered the shear stress. To maintain wall shear stress and diameter, viscosity should be increased. Wit, Schfer, Bismarck, Bolz, and Pohl (1997) and Tsai, Friesenecker, McCarthy, Sakai, and Intaglietta (1998) confirmed the beneficial effect of perfusion with high M_w dextran solutions as viscous plasma expanders. However, high viscosities will clog blood flow and carry oxygen to tissues. From the viewpoint of the relationship between the viscosity and blood flow rates, the viscosity values of CLI1 and CLI2 are appropriate and close to that of blood, whereas CLI3 may result in lung edema as reflected by the lung biopsy, possibly due to its high viscosity.

COP is related to the efficacy of colloidal volume expansion and is beneficial for blood flow recovery in resuscitation (Tonnessen, Tollofsrud, Kongsgaard, & Noddeland, 1993). Recently, PEGylation of BSA has been developed to yield oncotic properties similar to those of HSA but at a much lower concentration (Hangai-Hoger et al., 2006; Wettstein et al., 2004). Interestingly, CLI2 also shows an exceptionally higher COP than other groups (Fig. 1A) and thus less CLI2 was needed. Therefore, CLI2 is effective at a lower concentration like PEG-BSA and potentially has a better pharmacodynamic profile.

A principle criterion for rating the efficacy of plasma expanders is the ability to maintain the circulatory volume, which is determined by average M_w, M_w distribution, oncotic pressure, threshold for renal elimination, etc. (Mcilroy & Kharasch, 2003; Vercueil, Grocott, & Mythen, 2005). Polygelatin is rapidly metabolized by renal filtration for its small M_w (Persson & Grande, 2005). HES130 is more effective than HES70 as a volume replacement therapy in a canine hemorrhagic shock model (Kobori, Negishi, Nagai, & Iyama). Accordingly, when the hydrodynamic radius of the macromolecule (e.g., CLI1, 2.9 nm) is lower than threshold for renal elimination, it will be cleared. In contrast, those with a larger hydrodynamic radius (e.g., CLI2, 6.3 nm; CLI3, 8.2 nm; HSA, 3.5 nm; HES, 4.9 nm) will be maintained in the blood. Accordingly, the MAP of CLI1 and NS with a small M_w and a dynamic radius decreased in 2 h after transfusion. The CLI2 and CLI3 were maintained in the blood because the hydrodynamic radius was larger than the renal threshold value, resulting in a longer circulation time and less renal excretion.

The side effects on kidneys and lungs were M_w-dependant. After infusion, the plasma expander with M_w lower than the renal threshold will pass through the glomerulus, while those with a larger than renal threshold will stay in blood for a long time and lead to residues in organs. Residues have been found in the kidney biopsy of the CLI3 group, although no significant difference between animal tissue biopsy of transfusion of CLI1 and CLI2 and that of HES130 and HSA were found. In contrast, transfusion of NS solution resulted in serious damage to the lung and kidney, e.g., edema of renal collecting ducts, which agreed well with the literature (Rackow et al., 1983; Zornow, Scheller, Todd, & Moore, 1988). Presumably, the crystalloid property of NS solution may favor fluid shifting from the intravascular to the interstitial compartment, thereby increasing the risk of edema formation.

Side effects upon infusion are an important criterion of a plasma expander. A maximum dosage has been prescribed in the use of plasma expanders because of their side effects (Treib, Baron, Grauer, & Strauss, 1999). Moreover, adverse effects, such as pruritus and bleeding, occur frequently after chronic administration of 6% HES and are dose-dependent (Messmer, 1987; Frese et al., 2000). In contrast, 3% CLI2 can reduce the side effects because of its much lower concentration, as reflected by the biopsies of heart, liver, spleen, kidney, lung, and brain of bleeding animals.

In summary, the physiological properties of CLI with different M_w have been evaluated *in vivo* and *in vitro*, in comparison to HES, NS and HSA. The present study suggests that the exceptionally high COP of CLI2 improves the volume-expanding efficacy, the intravascular persistence, the hemodynamic and hemorrheologic properties of bleeding animals. Noticeable *in vivo* side effects (e.g., circulatory failure and functional disorders) in animals have not been observed. Thus, CLI2 can act as an efficient plasma substitute. Its physiological features are close to PEG-BSA, but it is inexpensive, which may make it more favored in clinical applications. Future work will focus on the *in vivo* metabolism of CLI2 and plasma coagulation upon transfusion with CLI2.

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