Conditions for Storage of Stroma-Free Hemoglobin Solutions for Fabricating Blood Substitutes

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Received 28 Jun 2003; Accepted 14 Oct 2003

Abstract

The stroma-free hemoglobin (Hb) solutions used for fabricating blood substitutes must be stable for a long period of time during storage and be available when massive clinical transfusions are required. One of the major problems in the long-term storage of Hb is its susceptibility to autoxidation, and thus becomes denatured (methemoglobin, metHb) and loses its capability to bind oxygen reversibly. In the study, porcine stroma-free Hb solutions were prepared by the aqueous two-phase system. The effects of varying conditions (temperature, pH, Hb concentration, cryoprotectant, and frequency of freeze-and-thaw process) on the extent of metHb formation during storage of Hb solutions were investigated. The results demonstrated that the stroma-free Hb solution obtained by the aqueous two-phase system was highly pure. The stroma-free Hb solutions can be safely stored at 4℃ or –20℃ without denaturation only for 2 months. Afterward, deterioration of Hb started to occur as evidenced by the increase in the metHb content. The extent of metHb formation increased significantly when the pH value of the storage solution is lower than the isoelectric point of Hb. Additionally, the extent of metHb formation increased with increasing the Hb concentration. A higher frequency of freeze-and-thaw process induced a greater extent of metHb formation. However, addition of cryoprotectant in the storage solution may inhibit the extent of metHb formation. In contrast, the stroma-free Hb solution can be safely stored at –80℃ without formation of metHb for at least 6 months.

***Keywords*:** Stroma-free hemoglobin, Storage condition, Blood substitute, Cryoprotectant

Introduction

Hemoglobin (Hb) has been used as raw materials for manufacturing blood substitutes [1-3]. However, because of its high oxygen affinity (P_{50} ~14 mm Hg) and short vascular retention time (~2 h), limitations on Hb as a blood substitute in the clinical therapy have been reported in the literature [4-6]. To decrease its oxygen affinity, Hb has been modified by pyridoxylation and followed by polymerization with glutaraldehyde [7-11]. It was reported that the polymerized Hb showed a P_{50} value of 19 to 22 mm Hg. Nevertheless, the reaction rate of Hb with glutaraldehyde is too fast to control its molecular weight distribution [4]. Hence, polymerization of Hb by glutaraldehyde is usually undertaken at 4℃ [4], a significant increase in the manufacturing cost. Additionally, the glutaraldehyde-polymerized Hb is relatively unstable and may release glutaraldehyde residues during storage or sterilization[4]. It was reported that glutaraldehyde is cytotoxic even at low doses [4]. This may impair the biocompatibility of the polymerized products.

In an attempt to overcome the aforementioned problems, a naturally occurring crosslinking agent, genipin, was used by our group to polymerize Hb [12]. The results indicated that the rate of Hb polymerization by glutaraldehyde was significantly faster than that by genipin and it readily produced polymers with molecular masses greater than 500000 daltons. It was found that the maximum degree of Hb polymerization by genipin was approximately 40% if over-polymerization is to be prevented. With increasing the reaction temperature, Hb concentration, and genipin-to-Hb molar ratio, the duration taken to achieve the maximum degree of Hb polymerization by genipin became significantly shorter. The P_{50} value of the unmodified Hb was 9 mm Hg, while that of the genipin-polymerized Hb increased to 21 mm Hg. It was found in a rat model that the genipin-polymerized Hb resulted in a longer circulation time than the unmodified Hb.

Hb consists of four polypeptide chains and four nitrogen-containing cyclic organic molecules called hemes

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[13]. The protein of Hb is composed of two identical polypeptide α-chains and two identical polypeptide β-chains. Each of the four polypeptide chains binds to a heme group that is then bonded to a central iron ion. Each iron is capable of binding one oxygen molecule, thus Hb binds a total of four oxygen molecules. To release oxygen, the Hb molecule undergoes a conformational change with 15^{o} rotation. The presence of the cofactor, 2,3-diphosphoglycerate (2,3-DPG), facilitates this conformational change. Thus, in the presence of the 2,3-DPG, Hb can release oxygen more readily at a higher tissue oxygen tension [14].

One of the major problems in the long-term storage of Hb is its susceptibility to autoxidation. Upon binding oxygen, the reduced form of heme iron (ferrous, Fe^{2+}) can react with oxygen to form ferric (Fe^{3+}) iron and superoxide [15]. When the heme iron is oxidized to the ferric form, Hb is denatured (methemoglobin, metHb) and oxygen can no longer bind reversibly. Thus, metHb is nonfunctional as an oxygen carrier [15]. Factors that may affect the formation of metHb in the preparation of the Hb solution include temperature, pH, Hb concentration, cryoprotectant, and frequency of freeze-and-thaw process.

In order to prepare stroma-free Hb solutions for fabricating blood substitutes and thus meet the broadly based need for supplies when massive fluid therapy is necessary, Hb must be stable during long-term storage [16,17]. In the study, porcine stroma-free Hb solutions were prepared by the aqueous two-phase system [18,19]. The effects of conditions (temperature, pH, Hb concentration, cryoprotectant, and frequency of freeze-and-thaw process) on the extent of metHb formation during storage of Hb solutions were investigated.

Materials and Methods

Preparation of stroma-free Hb solutions

Porcine stroma-free Hb solutions were prepared by the aqueous two-phase system as described in the literature [18,19]. The porcine blood was collected from a local slaughterhouse into glass bottles containing sodium citrate solution (3.7 g/dl). The bottles were kept in ice to minimize the formation of metHb. Upon return, the plasma was removed via centrifugation at 5000 × g for 10 min. The red blood cells were washed three times with normal saline (1:3 v/v) and lysed by treatment with three volumes deionized water, a hypotonic solution, over night. Subsequently, the cell membrane remnants were removed via centrifugation at 15000×g for 1h.

The separation and purification of stroma-free Hb was performed by the aqueous two-phase system. The resulting solution was dialyzed three times against a 0.05M phosphate buffered saline (PBS, pH 7.4), and the Hb was concentrated by ultrafiltration to 10 grams per deciliter. The Hb solution was subsequently sterilized by filtration through a 0.22-μm Millipore filter. The purity of stroma-free Hb was checked by electrophoresis in native-polyacrylamide gels (PhastSystem™, Pharmacia Biotech, Uppsala, Sweden) and by the gel filtration analysis using a high-performance liquid chromatographer (HPLC) equipped with a TSK G3000SWXL column (Tosoh Corp., Tokyo, Japan).

Storage conditions investigated

Effects of conditions (temperature, pH, Hb concentration, cryoprotectant, and frequency of freeze-and-thaw process) on the extent of metHb formation during storage of stroma-free Hb solutions were investigated. A 5 ml of sterilized stroma-free Hb solution was stored in a 10-ml vial for each of the studied condition (n = 3). The effect of temperature on the extent of metHb formation during storage of stroma-free Hb solutions was evaluated at: 4ºC, –20ºC, and –80ºC. A 6 g/dl of stroma-free Hb solution buffered with PBS at pH 7.4 was used. To elucidate the effect of pH value on the extent of metHb formation during Hb storage, a 6 g/dl of stroma-free Hb solution was buffered with PBS adjusted to pH 6.0, pH 7.4, or pH 8.5 by diluted HCl and NaOH at 4ºC, –20ºC, or –80ºC. To expose the effect of Hb concentration on the extent of metHb formation during Hb storage, a 6 g/dl, 12 g/dl, or 20 g/dl of stroma-free Hb solution buffered with PBS at pH 7.4 at 4ºC was used.

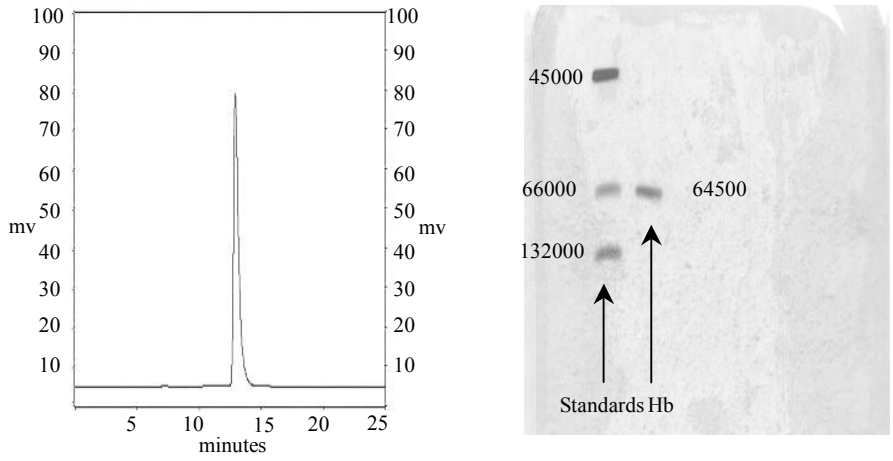
Additionally, the effect of addition of cryoprotectant (3% w/v glucose or sucrose) in the stroma-free Hb solution on the extent of metHb formation was investigated at 4ºC, -20ºC, or -80ºC. The entire duration for storage of stroma-free Hb solutions was 4~6 months. Samples of each studied group were taken out at distinct elapsed storage durations (with weekly analysis for the first month, then monthly) after initiation of the storage of stroma-free Hb solutions.

Moreover, the extent of metHb formation vs. the frequency of freeze-and-thaw process during storage of stroma-free Hb solutions with or without an addition of cryoprotectant (3% or 10% sucrose) was investigated at –20℃ or –80℃. Thawing of the frozen stroma-free Hb solution was performed by placing the storage vial in a water bath at 25℃. After thawing, the percentage of metHb formation was determined. Each freeze-and-thaw process took 1 day.

Hb and metHb concentration determination

The concentration of Hb was determined using the method described by Kilmartin [20]. First, dissolved 200 mg of $K_3Fe(CN)_6$, 50 mg KCN, and 140 mg of KH_2PO_4 in distilled water and made up to 1 liter with H_2O at a pH value 7.0~7.4. The solution was stored in a brown bottle. To 500 μl of above solution, added 20 μl of the Hb solution and 480 μl distilled water, let stand 30 min, then measured absorbance at 540 nm (Jasco V-530 UV/VIS Spectrophotometer, Tokyo, Japan) in a 1-ml cuvette. The calibration curve was made using commercially available Hb (Sigma Chemical Co., USA) at various known concentrations.

The concentration of metHb was measured as per the method described by Toshiyasu [21]. To microcells A and B, an aliquot (300 μl) of the sample was added. To microcells B, $K_3Fe(CN)_6$ solution (0.9 mol/l, 30 μl) was added and mixed; then the mixture was stood for 2 min. After water (300 μl) had been placed into microcells C, absorbance (D_1) of microcells A was measured at 563 nm (Jasco V-530 UV/VIS Spectrophotometer), with microcells C used as a reference. KCN solution (0.8 mol/l, 2μl) was then added to microcell A,



(a) (b)

Figure 1. (a) A typical elution pattern from the TSK G3000SW$_{XL}$ column for the purified stroma-free Hb solution; (b) Electrophoresis in native-page of the purified stroma-free Hb solution.

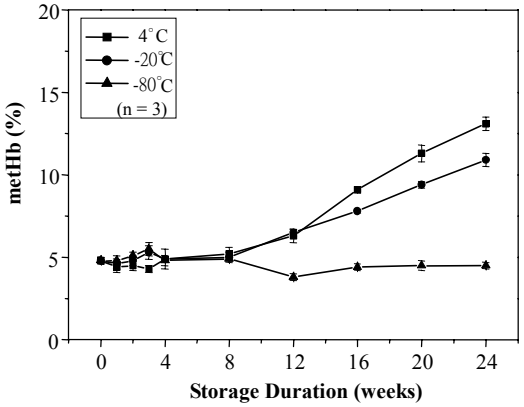


Figure 2. Percentages of metHb produced at distinct temperatures during the storage of Hb solutions.

and mixed gently with a pipette. After the microcell A had been stood for 30 s, the absorbance (D_2 of microcell A at the same wavelength was measured again, with microcell C as the reference. $K_3Fe(CN)_6$ solution (0.9 mol/l, 30 μl) was then added to C, which was then designated as microcell C'. Thereafter, the absorbance (D_3) of microcell B at the same wavelength was measured, with microcell C' used as the reference. Next, KCN solution (0.8 mol/l, 2 μl) was added to microcell B and mixed thoroughly. After the microcell had been stood for 30 s, the absorbance (D_4) of microcell B at the same wavelength was measured again, with microcell C' again used as the reference. The percent of metHb formation was calculated by the following equation:

$$\text{metHb (\%)} = (D_2 - D_1)/1.1(D_4 - D_3) \times 100\% \quad (1)$$

where the number (1.1) in the equation is the dilution factor [21].

Statistical analysis

Statistical analysis for the determination of differences in the measured properties between groups was accomplished using one-way analysis of variance and determination of confidence intervals, performed with a computer statistical program (Statistical Analysis System, Version 6.08, SAS Institute Inc., Cary, North Carolina, USA). All data were presented as a mean value with its standard deviation indicated (mean ± SD).

Results and Discussion

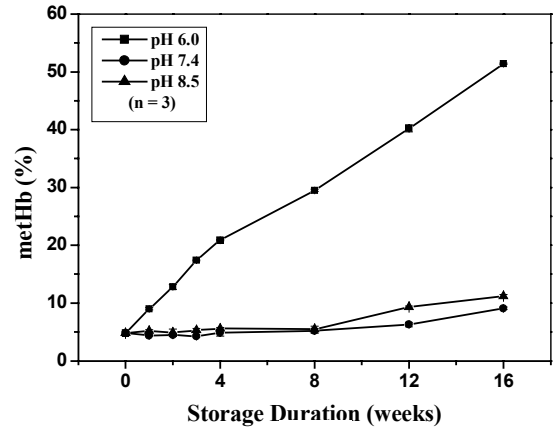
The stroma-free Hb solutions used for fabricating blood substitutes must be stable for a long period of time during storage and be available when massive clinical transfusions are required [16,17]. The study was designed to investigate the effects of varying conditions (temperature, pH, Hb concentration, cryoprotectant, frequency of freeze-and-thaw process) on the metHb formation during Hb storage. It was intended to minimize the extent of metHb formation while storing of stroma-free Hb solutions.

Preparation of stroma-free Hb solutions

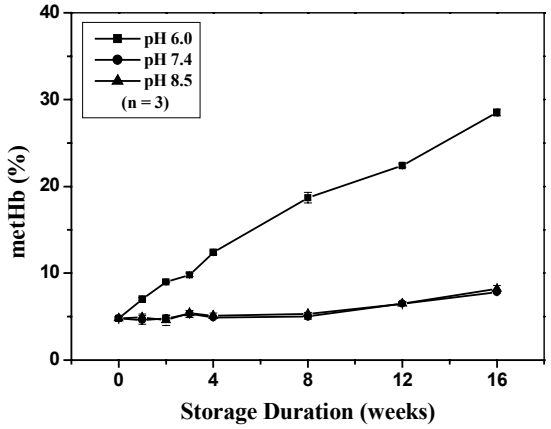
Extensive efforts have been made in the development of simple methods in the preparation of stroma-free Hb solutions from mammalian erythrocytes. In the study, the purified stroma-free Hb solution was obtained by the aqueous two-phase system. A typical elution pattern from the TSK G3000SWXL column for the purified stroma-free Hb solution is shown in Figure 1(a). The stroma-free Hb solution was eluted from the column as a single peak corresponding to a molecular weight of 64500 daltons. Electrophoresis in native-page of the purified stroma-free Hb solution yielded a single band corresponding to the 64500 daltons Hb (Figure 1(b)). These results demonstrated that the stroma-free Hb solution obtained by the aqueous two-phase system was highly pure.

Effects of temperature on the metHb formation

Figure 2 shows percentages of metHb produced at distinct temperatures during storage of stroma-free Hb solutions. The results indicated that at 4℃ and –20℃, the amounts of metHb formation remained unchanged until at 2-month after initiation



(a)



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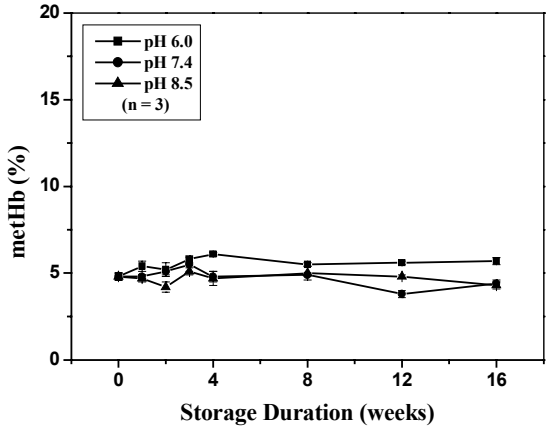


(c)

Figure 3. Percentages of metHb produced at distinct pH values during the storage of Hb solutions at (a) 4℃, (b) –20℃, and (c) –80℃.

of the storage of stroma-free Hb solutions ($p > 0.05$). Afterward, deterioration of Hb started to occur as evidenced by the increase in the amount of metHb formation ($p < 0.05$). This phenomenon was more prominent when the stroma-free Hb solution was stored at 4℃ than at –20℃. In contrast, the percentage of metHb formation stayed approximately the same throughout the entire course of the study during storage of the stroma-free Hb solution at –80℃ ($p > 0.05$). These results indicated that the extent of metHb formation decreased with decreasing the storage temperature. This fact

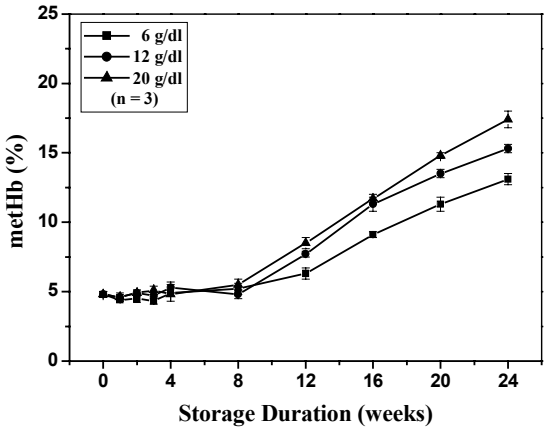


Figure 4. Percentages of metHb produced at distinct concentrations of Hb during storage at 4℃.

may be because with decreasing the storage temperature, the internal energy of the oxygen and Hb molecules decreased that may slow down the interaction between oxygen and Hb molecules and thus reduce the formation of metHb.

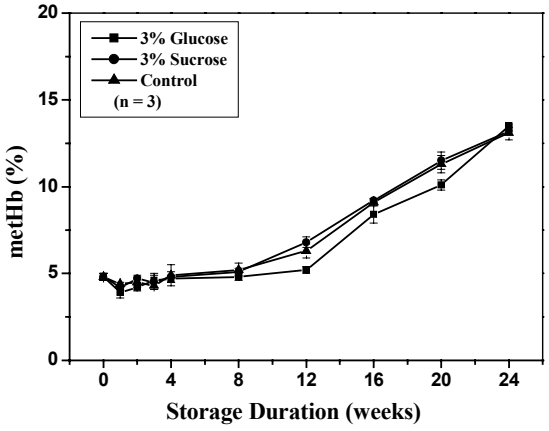
Effects of pH value on the metHb formation

Figure 3 presents percentages of metHb produced during storage of the stroma-free Hb solutions at 4℃, –20℃, and –80℃ under various pH values (pH 6.0, pH 7.4, and pH 8.5). As shown at 4℃ and –20℃, the percentages of metHb formation increased significantly with increasing the storage duration at pH 6.0 ($p < 0.05$); however, at pH 7.4 and pH 8.5, the extents of metHb formation did not change significantly until at 2-month after initiation of the Hb storage. At –80℃, the amounts of metHb formation did not change significantly throughout the entire course of the study ($p > 0.05$) for all the cases studied (at pH 6.0, pH 7.4, and pH 8.5).

It is known that the isoelectric point of normal Hb is at pH 6.8 [22]. Therefore, when the pH value of the stroma-free Hb solution is below 6.8, the conformation of Hb is unstable, even at low temperature (e.g., 4℃ or –20℃). Previous studies conducted by Brown and Mebine demonstrated that the rate of autoxidation of proteins increases dramatically with a decrease pH [15]. Therefore, the metHb formation at pH 6.0 was more remarkable than at pH 7.4 and pH 8.5. In contrast, at –80℃, the Hb is rather stable even at pH 6.0. This fact may be attributed to that the internal energy of Hb at –80℃ is too low to change its conformation as discussed before.

Effects of Hb concentration on the metHb formation

Figure 4 gives percentages of metHb produced with stroma-free Hb solutions stored at distinct Hb concentrations (6 g/dl, 12g/dl, and 20 g/dl) at 4℃. The results indicated that percentages of metHb remained unchanged for 2 months for all studied cases ($p > 0.05$). Subsequently, deterioration of Hb started to occur as evidenced by the increase in the metHb content. This phenomenon was more prominent as increasing the Hb concentration ($p < 0.05$). Multimerization of Hb to dimmers (i.e., dimerization of Hb molecules) can occur through disulfide cross-linkage of the β globin (β-chain) Cys93 residues when the Hb concentration is high [10]. This may denature Hb and thus increase the formation of metHb.



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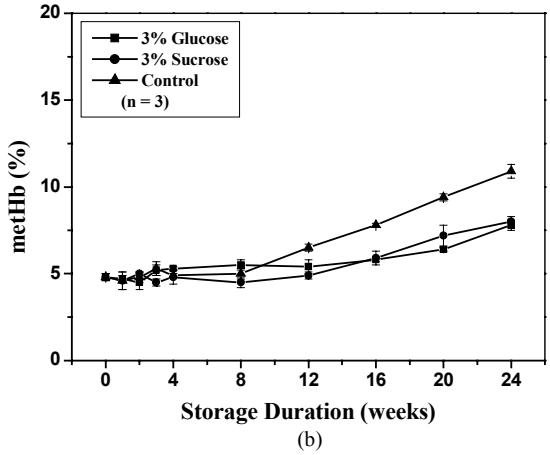

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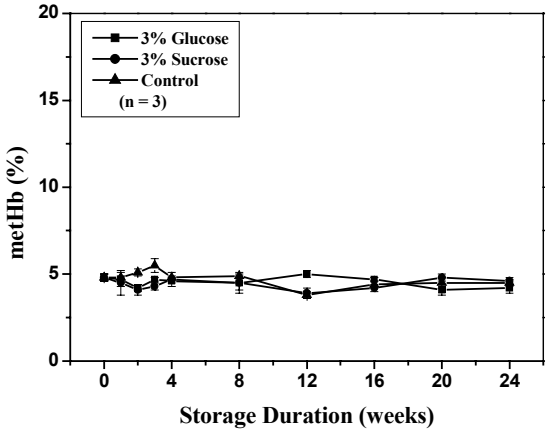


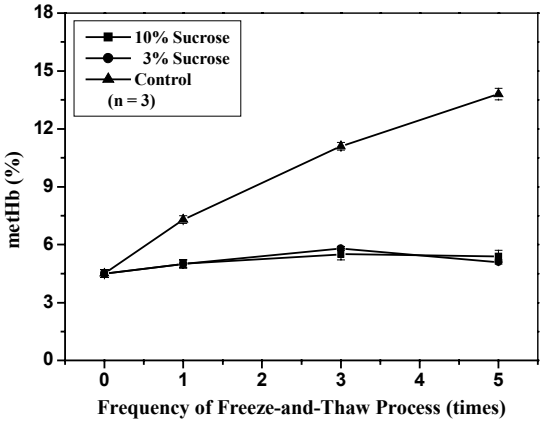
(c)

Figure 5. Percentages of metHb produced with and without (control) an addition of cryoprotectant (3% glucose or sucrose) at (a) 4℃, (b) –20℃, and (c) –80℃.

However, addition of cryoprotectant in the storage solution may decrease the rate of multimerization and stabilize the conformation of Hb [15].

Effects of cryoprotectant on the metHb formation

Figure 5 presents percentages of metHb produced during storage of stroma-free Hb solutions at 4℃, –20℃, and –80 ℃ with or without (control) an addition of cryoprotectant (3% glucose or sucrose). It is known that glucose and sucrose, as a cryoprotectant, may help maintain the native structures of proteins during long-term storage [23,24]. This fact was



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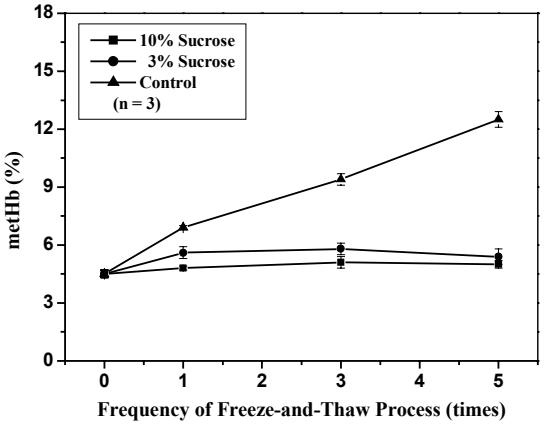


(b)

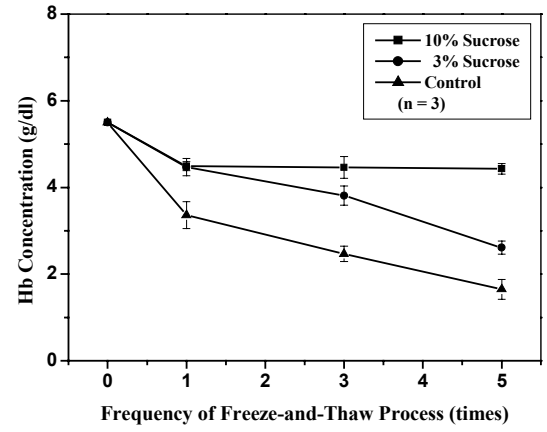
Figure 6. Percentages of metHb produced vs. frequency of freeze-and-thaw process during storage of stroma-free Hb solutions with or without (control) an addition of cryoprotectant (3% or 10% sucrose) at pH 7.4 at (a) –20 ℃ and (b) –80℃.

demonstrated in the study for the stroma-free Hb solution stored at –20℃. As shown in Figure 5(b), at –20℃, the amounts of metHb produced with an addition of cryoprotectant (glucose or sucrose) were significantly less than that without an addition of cryoprotectant (control, $p < 0.05$).

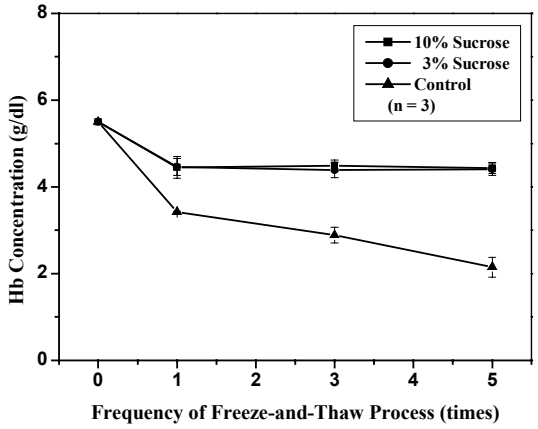
However, the effect of temperature on the metHb formation was more pronounced than that of cryoprotectant when the stroma-free Hb solutions were stored at 4℃ and -80 ℃. At 4℃ (Figure 5(a)), percentages of metHb formation remained unchanged for approximately 2 months. After that, deterioration of Hb started to occur as evidenced by the increase in the metHb content. No significant differences were observed with or without an addition of cryoprotectant (glucose or sucrose, $p > 0.05$). At -80℃, the amounts of metHb formation stayed about the same throughout the entire course of the study for all cased studied with or without an addition of cryoprotectant ($p > 0.05$).

Effects of frequency of freeze-and-thaw process on the formation of metHb

Figure 6 shows the effect of frequency of freeze-and-thaw process on the formation of metHb with an addition of sucrose in a concentration of 0% (control), 3%, or 10% in the storage



(a)



(b)

Figure 7. Variation of Hb concentrations vs. frequency of freeze-and-thaw process during storage of stroma-free Hb solutions with or without (control) an addition of cryoprotectant (3% or 10% sucrose) at pH 7.4 at (a) –20℃ and (b) –80℃.

solution at –20℃ and –80℃. As shown, without an addition of sucrose, the formation of metHb increased significantly with increasing the frequency of freeze-thaw process both at –20℃ and –80℃ ($p < 0.05$). However, the formation of metHb was significantly inhibited by an addition of 3% or 10% sucrose in the storage solution ($p < 0.05$).

The reason which resulted in a greater extent of metHb formation, without an addition of cryoprotectant at –20℃ or –80℃, can be attributed to the process of freezing and thawing. It is known that freezing and thawing may denature proteins [24,25]. The denaturation of Hb during the process of freeze-and-thaw may be due to the crystallization of solvent water, which would lose the hydrophobic bonds of the Hb molecules and break the intramolecular hydrogen bonds [25,26]. Therefore, a higher frequency of freeze-and-thaw process induced a greater extent of metHb formation. With an addition of cryoprotectant (3% or 10% sucrose), the metHb formation was significantly inhibited ($p < 0.05$).

It is known that denaturation of Hb during the process of freeze-and-thaw is largely irreversible and often takes the form of rapid aggregation [15] and thus decreases the Hb concentration. Figure 7 shows the Hb concentration vs. the frequency of freeze-and-thaw process at –20℃ and –80℃ with or without an addition of sucrose. As shown, the Hb concentration significantly decreased after the first freeze-and-thaw process at –20℃ and –80℃. However, the decrease in the Hb concentration with an addition of sucrose was significantly inhibited ($p < 0.05$).

Conclusions

In conclusion, the aforementioned results indicated that temperature, pH, Hb concentration, and frequency of freeze-and-thaw process can all significantly affect the formation of metHb during storage of stroma-free Hb solutions. However, an addition of cryoprotectant may inhibit the extent of metHb formation.

Acknowledgments

This work was supported partly by a grant from the Veterans General Hospital, Tsing-Hua, and Yang-Ming Joint Research Program (VTY-91-P4-23) and partly by another grant from the National Science Council of Taiwan, Republic of China (NSC-91-2320-B-007-004).

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