HYPOTENSIVE EFFECTS OF STEMMA-FREE HAEEMOGLOBIN SOLUTIONS ATTRIBUTABLE TO
ADENINE NUCLEOTIDES

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Short title: Adenine nucleotides in haemoglobin solutions

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SUMMARY

Aqueous solutions of stroma-free human-haemoglobin are being evaluated as potential oxygen-carrying resuscitation fluids. However, there are indications that such solutions may produce toxic side-effects in vivo. Stroma-free haemoglobin solution produced a 50% fall in mean arterial pressure when infused into a small animal model despite containing very low levels of non-haem protein and phospholipid contaminants. This effect was not produced by haemoglobin solutions after extensive dialysis. Red cell-derived adenine nucleotides were found to be present in concentrations high enough to cause such a response (80-85μg/ml). We have developed a chromatographic assay capable of predicting hypotension in our animal model and consider that the complete absence of adenine nucleotides must be confirmed in all studies concerning the possible toxic side-effects of stroma-free haemoglobin solutions.

Key words: Stroma-free haemoglobin, blood substitute, adenine nucleotides
INTRODUCTION

Considerable research is now being devoted to the development of modified stroma-free haemoglobin (SFHb) solutions as potential oxygen-carrying plasma expanders (DeVenuto and Zegna, 1983; Seghal et al., 1983; Keipert et al. 1989). The near-normal oxygen affinity, long shelf life and potentially reduced risk of disease transmission make such solutions uniquely suitable in situations where blood transfusion is not practical. More conventional resuscitative fluids such as albumin, hydroxyethyl starch (HES) and Ringers' lactate lack significant oxygen-carrying capacity.

However, haemoglobin (Hb) solutions have been found to cause vasoconstriction in coronary, cerebral and renal vascular beds (Tanishima 1980; Vogel et al. 1986; Lieberthal et al. 1987; Biro et al. 1988). There are also early reports that Hb solutions, of varying degrees of purity, could produce an overall hypotensive response in intact animal models (Parry 1988).

As part of a programme to develop a modified SFHb solution for clinical use we infused Hb solution, from various stages in our production process, into a small animal model designed to evaluate vasoactivity in blood products (MacGregor et al. 1989). Infusion of a small volume of SFHb, prior to chemical modification, produced a significant fall in mean arterial pressure (MAP). This response was almost totally absent when the same volume of extensively dialysed SFHb was infused and would therefore suggest that the factor(s) responsible must be of low molecular weight (less than 10kd). In view of the low levels of non-haem protein and residual stromal phospholipid contaminants present in our SFHb, a vasoactive response on this scale was unexpected.
Adenyl nucleotides have been known to be extremely potent hypotensive compounds \textit{in vivo} since the work of Drury and Szent-Györgyi (1929) and we suspected that red cell-derived adenyl nucleotides might be present and responsible for at least part of the hypotension produced \textit{in our animal model}. We have therefore investigated this possibility by correlating the degree of hypotension produced by a given haemoglobin solution with the concentration of adenyl nucleotides present, as measured by a fast protein liquid chromatographic (FPLC) assay specifically devised for this purpose.
MATERIALS AND METHODS

Preparation of Haemoglobin Solutions

Eight to ten units of outdated human red cells were washed four times with isotonic saline (IBM 2991 cell washer). Six volumes of packed cells were then lysed by addition of four volumes of sterile, deionised water. For comparison of adenine nucleotide contamination Hb solution was prepared by two methods. In the first, stroma was removed by ultrafiltration using a 0.22µm hollow fibre unit (MiniKros: Northumbria Biologicals). In the second, the lysate underwent two chloroform extractions (6 volumes red cells:4 volumes chloroform). The chloroform, stromal and haemoglobin layers were separated by centrifugation (3000g; 20 minutes). The osmolality of the Hb solution, prepared by both methods, was then adjusted to 290mOsm/kg by addition of solid NaCl, passed through a sterile 0.22µm filter (Gelman Sciences) into transfer packs (Pl 1240 Femwal) and stored at -60°C until use. For comparison in the animal model, aliquots of this Hb solution were subjected to extensive dialysis against normal saline before sterile filtration and storage as described.

Polymerisation and Pyridoxalation

All operations were carried out at 4°C. The Hb solutions were concentrated to 140g/L using a hollow fibre dialysis cartridge (10kD pore size; Cobe Centrisystem 400) with an applied back pressure of 55kPa and deoxygenated by gas exchange with nitrogen using a blood gas exchanger (SciMed Life Systems Inc.). The polymerisation step may be carried out in
either the oxygenated or deoxygenated state. We therefore found it convenient to deoxygenate whilst the polymerisation step was proceeding in order to ensure that the final concentration of oxyHb was less than 2% before proceeding to pyridoxalation. Polymerisation was carried out using glycolaldehyde (Sigma). A 10% stock solution of glycolaldehyde was prepared in 50mM sodium phosphate buffered saline pH 7.4. In our final procedure, 5.26ml of this stock solution was added per 100ml of 140g/L Hb solution to give a final molar ratio of 40:1 glycolaldehyde/Hb. Polymerisation was monitored by FPLC gel filtration (Superose 12 column: Pharmacia). After 5-6 hours pyridoxal 5' phosphate (PLP) was added in a modification of the method of DeVenuto and Zegna (1983). In our final procedure, a 4.08% solution of PLP was prepared in 1M Tris buffer pH 7.4, and 5ml of this solution added per 100ml of 140g/L solution to give a molar ratio 4:1 PLP/Hb. Both reactions were allowed to proceed for a further 30 minutes.

In order to stabilise Hb-PLP covalent bonds and terminate the polymerisation process, sodium borohydride (NaBH₄; Sigma) was then added. A 3.12% solution was prepared in 10⁻³ M NaOH; 5ml of this solution were added per 100ml of 140g/L Hb solution to give a molar ratio of 20:1 NaBH₄/Hb and left overnight at 4°C. Air was then readmitted to the reaction vessel and the Hb solution dialysed extensively against deionized water using a kidney dialysis cartridge (pore size 10kD) to remove excess reagents and red cell-derived low molecular weight contaminants. Simultaneous concentration to 140-160g/L was achieved by applying a back pressure of 55kPa and the solution finally dialysed against Krebs' solution pH 7.4, before 0.22μm filtration (Gelman Sciences) into sterile transfer packs and storage at -40°C.
In Vitro Characterisation

Hb, oxyHb and methHb concentrations were measured using an OSM 3 Hemoximeter (Radiometer, Copenhagen). Total protein was measured by refractometry and non-haem protein was obtained as the difference between total protein and Hb concentration. Colloid osmotic pressure (COP) was measured using a colloid osmometer (type 050; Conotec). Oxygen affinity (P_{50}) was measured under standard conditions (P_{CO2} = 5.33kPa; pH 7.4; 37°C) using a Hemox Analyser Model B (TCS Medical Products Company). Relative molecular weight distribution was estimated by FPLC gel filtration (Superose 12 column; Pharmacia). Endotoxin was estimated by limulus lysate gel clot assay (Pyrogen kit; Whittaker Biologicals). Osmolality was determined by freezing point depression (3MO Osmometer; Advanced Instruments Inc.).

For assessment of residual stromal phospholipids, lipids were extracted by 2:1 (v/v) chloroform/methanol extraction, concentrated 100-fold by rotary evaporation and spotted onto precoated silica gel thin layer chromatography (TLC) plates (Kieselgel; Merck). Plates were developed in a tank containing 100ml hexane:diethylether:formic acid 80:20:2 (v/v/v). Phospholipids, which remain at the origin, were determined after saponification by gas liquid chromatography (GLC) as fatty acid methyl esters (Phillips GLC; flame ioniser detector). The column was packed with 10% SP 2330 on Chromasorb W-AM-Supelco. The temperature programme was as follows:- Column 180°C - held for 3 minutes; ramp +3°C/minute to 250°C -held for 5 minutes. Injector and detector temperatures were 220°C and 300°C respectively.
Vasoactivity Testing in the Rat Model

Adult, female Sprague-Dawley rats (250g) were anaesthetised with sodium pentobarbitone (Sagatal; May and Baker) at a dose of 60mg/kg intraperitoneally. The animals were cannulated via the jugular vein for infusion of the test solutions and via the femoral artery for monitoring of MAP and heart rate. The cannulae had previously been flushed with heparinised saline; the jugular cannula was then used for infusion of heparin to 100U/kg body weight. Hb solutions were infused as a bolus (0.58ml/12sec) and the responses compared with those given by similar bolus infusions of both albumin and saline. The animals were sacrificed at the end of the experiment by an intravenous lethal dose of Euthatal (May and Baker). All procedures conformed to regulations laid down in the Animals (Scientific Procedures) Act 1986 of the United Kingdom.

Adenyl Nucleotide Assay

The low molecular weight fraction was removed from Hb solutions by ultrafiltration against a flat membrane with a 10kD cut-off (Amicon PM10 membrane). 1ml of this clear filtrate was then diluted to 50ml in sterile, deionised water and applied to an FPLC anion exchange column (Mono Q 10/10; Pharmacia) using a 50ml Superloop attachment. This dilution step allowed nucleotides to bind in a salt-free environment. Guanosine triphosphate (GTP; 20µg; Sigma) was added as an internal standard. The eluants were chosen so as to allow testing of individual fractions for vasoactivity in the animal model. Eluant A was deionised water and eluant B was 0.1M sodium phosphate, 0.9M NaCl pH 7.0. The appropriate gradient for
separation of the nucleotides was chosen experimentally. Initially, ATP concentration was also assayed by luminometry (ATP assay kit; LKB) in order to validate the FPLC assay (n = 10). Data were analysed using the Paired Student T Test.
RESULTS

The fall in MAP that is typically produced by a bolus infusion (0.58 ml) of undialysed SFHb, produced by filtration, is shown in Figure 1(a). The reduced responses obtained with 10-fold and 100-fold dilutions are shown in Figures 1(b) and (c) respectively. The hypotension was also accompanied by a transient bradycardia (10-20%) which persisted for 30-60 seconds following infusion (not shown). Anaphylaxis was not seen. SFHb solution produced by chloroform extraction was not tested in the animal model at this stage due to the presence of residual chloroform.

A typical FPLC adenyl nucleotide elution profile obtained from undialysed SFHb solution following chloroform extraction is shown in Figure 2. It is necessary to separate Hb from nucleotides by ultrafiltration before applying the sample to the anion exchange column because, under these experimental conditions, Hb will bind and elute at the same position as AMP.

The ten samples in which ATP was also assayed by luminometry showed no significant difference at the 0.05 level of confidence (t=0.47), thereby confirming the accuracy and reliability of the FPLC assay. Nucleotide levels in SFHb produced by the two preparative techniques are given in Table 1.

Figure 3 shows dose response curves obtained in the rat model by SFHb produced using ultrafiltration (upper curve) and the negligible response produced when this same solution has been extensively dialysed to remove all low molecular weight contaminants (lower curve). Similar bolus infusions of albumin and saline typically produced a 3-5% hypotension. The third curve shows the effect produced when adenyl nucleotides (Sigma) are
then added to the dialysed SFHb solution, to the final concentrations given for SFHb(1) in Table 1, in an attempt to mimic the original response. Adenosine was not included due to the qualitative nature of the assay for this nucleoside. It should be noted that not only was the response magnitude restored by adding nucleotides but also the time course was similar to undialysed SFHb.

Figure 4 shows the fall in MAP produced when SFHb solution was tested at three different points in our production process. It is clear that dialysis has significantly reduced, but not completely eliminated, the hypotensive response in the three batches tested. It would also appear that subsequent chemical modification has introduced no adverse effects.

The in vitro characteristics of SFHb solutions produced by filtration and chloroform extraction are given in Table 2. Comparative values for polymerised SFHb (poly-SFHb) are also listed. The total Hb concentration in poly-SFHb was 140-160g/L, compared with 70g/L for the unpolymerised SFHb solutions. However, because the degree of polymerisation was deliberately low, the COP is only slightly reduced. The methHb concentration is higher in poly-SFHb (8-10%), presumably due to the longer processing time involved.

Phospholipid levels ranged from 0.3mg/dl in the SFH solution produced by filtration, to 0.1mg/dl in the chloroform extracted Hb. The latter represents the lower limit of assay sensitivity.

The limulus lysate assay was positive for the presence of endotoxin in all three solutions. However, endotoxin infused into rats at a concentration a thousand-fold higher than the upper limit in our poly-SFHb solution has been reported to have produced no effect on systemic arterial pressure (Wehrly et al 1987).
DISCUSSION

This study demonstrates the potential for adenyl nucleotide contamination in SFHb solutions. Published reports concerning the toxic effects of SFHb, both modified and unmodified, involve a wide variety of preparative techniques (DeVenuto and Zegna 1983; Seghal et al. 1983; Keipert et al. 1989; White et al. 1986; Feola et al. 1988). In vitro characterisation generally includes assessment of residual stromal phospholipid, endotoxin and non-haem protein levels in the test solution. However, from our results it is clear that a SFHb solution may contain these contaminants at levels well below those which these workers have judged to be toxic in various animal models, and yet retain adenosine and adenyl nucleotides at concentrations high enough to cause a 50% fall in MAP in our rat model.

The endothelium is first in line of exposure to any blood borne toxic factors and plays a vital role in the regulation of vascular tone and haemostasis. The potent vasodilator properties of purines such as adenosine and the adenine nucleotides have been known since the work of Drury and Szent-Gyorgyi (1929). In addition, adenosine has recently been implicated in the pathogenesis of acute renal failure (Oken and Reilly 1989). Exogenously administered purines are rapidly degraded, both locally and during trans-pulmonary passage, and so it is now believed that in whole animal models, the magnitude of the hypotension closely reflects levels of arterial plasma adenosine (Sollevi and Fredholm, 1981). Figure 3 shows that when the three phosphorylated adenyl nucleotides are added to dialysed SFHb solution, in an attempt to mimic the original response, the resulting dose response curve lies slightly below that produced by undialysed SFHb solution. This finding is possibly due to the absence of extra adenosine
itself, which we judged it better to omit in this experiment due to the qualitative nature of the chromatographic assay for this nucleoside. However, the time course of the response is very similar to that produced by undialysed SfHb.

Ferrell and Koshbaten (1990) have reported that adenosine produces concentration-dependent relaxation in blood vessels of the rabbit knee joint, which is not dependent on the presence of an endothelial layer. These same authors have also found that ATP will produce a transient vasoconstriction followed by a longer lasting dilatation and that this effect is dependent on the presence of endothelium. ATP and ADP are known to cause release of the smooth muscle relaxant endothelium derived relaxing factor (EDRF) and prostacyclin release from endothelial cells (Delbro and Burnstock, 1987) although they report wide interspecies variability in the response to purines; in the rat adenosine and ATP can cause EDRF release whereas, in the dog, prostaglandins may mediate the adenosine-induced response.

Hb itself has been reported to produce various side effects in animal models, most notably vasoconstriction in a wide variety of whole animal and isolated organ experiments (Valeri et al 1987; Feola et al 1989; Connor and Feniuk 1987; Vogel et al 1987; Gilroy et al 1988). Much debate exists as to the mechanisms of Hb-induced vasoconstriction but it is well known that oxyHb is a potent inhibitor of EDRF (Martin et al 1986) and that both oxyHb and carboxyHb are more potent in their vasoconstrictor effects than either deoxyHb or metHb (Valeri et al 1987). This would suggest a possible role for superoxide radicals generated during autoxidation to metHb (Sadrzadeh 1987).

Hb also acts by mechanisms other than inhibition of EDRF to produce a
wide diversity of effects although the ultimate response to very pure Hb quite probably involves inhibition of EDRF and endothelin release (Yanagisawa et al. 1988), both causing vasoconstriction, and vasodilation resulting from prostacyclin release from endothelial cells. Clearly, the presence of adenyl nucleotides in the Hb test solution would introduce very serious complications and make the interpretation of toxic effects attributable to Hb alone almost impossible.

The levels of nucleotides present in SFHb solutions prepared by two methods are very similar (Table 1). It is also clear from our results that extensive dialysis will remove them from Hb solutions to levels below 1.0 μg/ml which is the lower limit of sensitivity of our chromatographic assay. In our current protocol, designed to produce poly-SFHb for eventual clinical use, it is convenient to leave this dialysis step until after the chemical modification steps are complete, so enabling excess reaction products to be removed at the same time. The fact that poly-SFHb causes a response no greater than that produced by dialysed SFHb, and comparable with albumin and saline controls, suggests that chemical modification per se has no adverse effects on vasoactivity. However, these findings may not explain the vasoconstrictor response reported in many isolated organ models perfused with Hb solutions already subjected to long periods of dialysis and it would seem probable that these effects are a direct effect of the Hb molecule itself (White et al. 1986).

In conclusion, we have demonstrated that unless a SFHb solution has been subjected to purification processes involving extensive dialysis, red cell-derived adenine nucleotides are retained at levels high enough to cause a significant hypotension in the rat model described. The evidence would suggest that studies with standardised, highly purified preparations...
are required and that the presence of vasoactive contaminants must be considered in all preparations deriving from red cells.

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LEGENDS TO FIGURES

Figure 1
The fall in MAP produced on bolus (0.5ml) infusion of undialysed SFHb solution into the anaesthetised rat model.
(a) infusion of neat SFHb solution (time course 60-70 seconds)
(b) infusion of 1/10 dilution in saline
(c) infusion of 1/100 dilution in saline

Figure 2
A representative FPLC anion exchange profile (Mono Q 10/10, Pharmacia) of adenyl nucleotides assayed in undialysed SFHb solutions prepared either by ultrafiltration or chloroform extraction. The peak eluting immediately after AMP is unidentified impurity which, when infused into the rat model, produces no vasoactive response (not shown). GTP (20μg) was the internal standard. Eluant A was deionised water and eluant B was 0.1M sodium phosphate, 0.9M NaCl pH 7.0.
Gradient 0 - 70% B in 70 minutes: flow rate 1.0ml/minute: absorbance 254nm
**Figure 3**

Dose response curves produced in the rat model by bolus (0.58ml) infusion of SFHb solutions before and after removal of low molecular weight component by dialysis.

Key:

(a) % fall (+S.D) produced on 0.58ml bolus infusions of logarithmic dilutions of undialysed SFHb solution (n=6).

(b) % fall (+S.D) upon similar infusion of SFHb solution following dialysis (n=7).

(c) % fall (+S.D) produced when adenine nucleotides have been added to dialysed SFHb solution in an attempt to reproduce the original hypotension (n=7). Adenosine was not included for reasons discussed in the text.

**Figure 4**

Percentage fall in MAP caused by SFHb solution tested at different stages of modification process during three consecutive batches.

Key:

SFHb = not dialysed

SFHb = extensively dialysed

Poly-SFHb solution (extensively dialysed)
**TABLE 1**

*Adenine Nucleotide Levels in Undialysed SFHb Solutions*

Concentrations are μg/ml±S.D (n=6 batches)

<table>
<thead>
<tr>
<th></th>
<th>SFHb(1)</th>
<th>SFHb(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>AMP</td>
<td>39.0±6.5</td>
<td>45.9±12.4</td>
</tr>
<tr>
<td>ADP</td>
<td>23.8±2.7</td>
<td>23.9±12.4</td>
</tr>
<tr>
<td>ATP</td>
<td>17.8±3.1</td>
<td>14.3±3.9</td>
</tr>
<tr>
<td>Total</td>
<td>80.6±5.9</td>
<td>84.1±13.6</td>
</tr>
</tbody>
</table>

Key: SFHb(1) prepared by filtration of lysate  
     SFHb(2) prepared by chloroform extraction of lysate
**TABLE 2**

**Characteristics of SFHb and Modified-SFHb Solutions**

<table>
<thead>
<tr>
<th></th>
<th>SFHb(1)</th>
<th>SFHb(2)</th>
<th>Poly-SFHb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin</td>
<td>70g/L</td>
<td>70g/L</td>
<td>140-160g/L</td>
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<td>Methaemoglobin</td>
<td>3-5%</td>
<td>3-5%</td>
<td>8-10%</td>
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<tr>
<td>Non-haem protein</td>
<td>2-4%</td>
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<td>(% of total protein)</td>
<td></td>
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</tr>
<tr>
<td>Osmolality</td>
<td>290mOsm</td>
<td>290mOsm</td>
<td>290mOsm</td>
</tr>
<tr>
<td>COP</td>
<td>4.67kPa</td>
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<td>4.0kPa</td>
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<tr>
<td>P&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1.2-1.6kPa</td>
<td>1.2-1.6kPa</td>
<td>2.9-3.2kPa</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>68kD</td>
<td>68kD</td>
<td>[70% 68kD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30% 130kD</td>
</tr>
<tr>
<td>Phospholipids</td>
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<td>&lt;0.1mg/dl</td>
<td>&lt;0.1mg/dl</td>
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<tr>
<td>Endotoxin</td>
<td>10-20IU/ml</td>
<td>10-20IU/ml</td>
<td>60-100IU/ml</td>
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<tr>
<td>Total nucleotides</td>
<td>80.6±5.9</td>
<td>84.1±13.6</td>
<td>&lt;1.0</td>
</tr>
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<td>(ug/ml+S.D.)</td>
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<tr>
<td>n=6 batches</td>
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</table>

**Key:**

SFHb(1) prepared by filtration of lysate

SFHb(2) prepared by chloroform extraction of lysate

Poly-SFHb is pyridoxalated, lightly polymerised and extensively dialysed SFHb(2)
Figure 2.