# 人工血液

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目　次



# ARTIFICIAL BLOOD

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Contents

# 第15回日本血液代替物学会年次大会

## The 15th Annual Meeting of the Society of Blood Substitutes, Japan

大会長： 堀之内 宏久（慶應義塾大学医学部呼吸器外科 准教授）

『血液代替物の開発戦略』

会期： 平成20年10月23日（木）

会場： 慶應義塾大学信濃町キャンパス　北里講堂

東京都新宿区信濃町35

## The 6th Current Issues on Blood Substitute Research

会長： 小林 紘一（慶應義塾大学 名誉教授）

Koichi Kobayashi（Keio University）

会期： 平成20年10月24日（金）・25日（土）

October 24－25，2008

会場： 慶應義塾大学信濃町キャンパス 大会議室・中会議室（新棟11階）

東京都新宿区信濃町35

Keio University Shinanomachi Campus
35 Shinanomachi, Shinjuku-ku, Tokyo Japan

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〒160-8582　東京都新宿区信濃町35
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# 年次大会　大会長　挨拶

このたびは第15回を迎えました日本血液代替物学会の年次大会をお世話することとなりました。今回の学会は小林紘一血液代替物学会会長が、２年に一度開催される、Current Issues on Blood Subutitute Research（CIBSR）を主宰されるのに合わせて合同学会として開催させていただくこととなりました。当初すべて英語でとも考えたのですが、日本語で自由にDisucussionができる時間もあったほうがよいとの意見もありましたので、23日を日本の血液代替物学会年次大会として、24、25日を第６回のCIBSRとして行うこととしました。

年次大会では輸血代替を目的とした研究に関する演題が多く集まりましたので、シンポジウムのセッションを「輸血代替としての人工酸素運搬体」として討論してゆきたいと思います。また、教育講演、招請講演はCIBSRへ移して、小林弘祐先生に「組織への酸素運搬について」およびZapol先生に修飾ヘモグロビンによる血圧上昇に対するNO吸入の効果について御講演いただくこととしました。

人工酸素運搬体は臨床応用が間近といわれながら実現への道筋が遠いようで、研究サイドで新たな知見を発表し、臨床応用への後押しができればと考えています。

一般会員のみならず、厚労省、日赤、企業の方のご参加もいただき、官産学にわたる交流ができればと期待しています。

多数のご参加を心からお待ちしております。

第15回日本血液代替物学会年次大会
大会長　堀之内 宏久
（慶應義塾大学医学部呼吸器外科）

# お知らせとお願い

**■会員・参加者の方へ**

＜開場及び受付開始＞

第15回年次大会およびThe 6th Current Issues on Blood Substitute Research（6th CIBSR）の開場時間は，10月23日（木）午前 9 時～，24日（金）・25日（土）午前 8 時～です．

＜参加登録＞

第15回年次大会の受付は23日（木）北里講堂前のみです．なお，事前受付は行いません．参加登録費は下記の通りです．

会員　　10,000円
非会員　　20,000円
学生　　5,000円（受付にて必ず学生証を提示してください．）

23日に第15回年次大会の参加登録を行った方は，追加登録なしで24・25日の6th CIBSRにご参加いただけます．

＜抄録集＞

抄録集は会員全員に事前送付しています．また当日は受付にて 1 部1,500円で販売いたします．

＜新入会受付＞

日本血液代替物学会に未入会の方は受付で入会手続きをおとりください．

年会費は正会員10,000円，購読会員6,000円，学生会員5,000円です．

＜懇親会＞

参加者相互の親睦を図るため，10月23日（木）午後 5 時30分よりレストラン オアシス（慶應義塾大学病院 新棟11階）におきまして懇親会を開催いたします．（終了: 午後 7 時30分頃）参加費は無料ですので，是非ご参加ください．

**■The 6th Current Issues on Blood Substitute Research（6th CIBSR）への参加について**

24日（金）からのご参加は下記参加登録費となりますのでご注意ください．

一般参加　　30,000円
同伴者　　15,000円
学生　　5,000円（受付にて必ず学生証を提示してください．）

**■演題発表される方へ**

1．本学会の発表はすべてPCプロジェクターで行いますのでご了承ください．
2．Windowsの場合（動画を含む場合を除く）はUSBフラッシュメモリーまたはCD-Rに保存してご提出ください．CD-Rについては，ハイブリッドフォーマットのみといたします．MacintoshおよびWindowsで動画を含むデータの場合は，ご自身のパソコンをお持ちください．なお，当日お持込になるPCまたはメディアのウイルスチェックは必ず事前に行ってください．スクリーンセーバー，ウイルスチェックならびに省電力設定はご発表前にあらかじめ解除しておいてください．静止画像はJPEG形式で作成されることをお勧めいたします．
3．事務局でご用意しておりますPC（Windows）には，OS: Windows VistaでPowerPoint 2003をインストールしており，PowerPoint 2000以降のバージョンには対応可能です．この環境にて正常に作動するデータをご用意ください．ご発表データは，会期終了後，事務局で責任を持って消去いたします．
4．当日はUSBフラッシュメモリー，CD-R等のバックアップデータを必ずご用意ください．当日のデータ及びバックアップとして使用させていただきます．
5．ご発表の 1 時間前までに受付にいらっしゃって，試写を行ってください．
6．ご自身のPCでの発表を希望される場合は，D-sub15ピンによるモニター出力が必要です．事務局でD-sub15ピンの接続ケーブルをご用意いたしますが，ご持参いただくPCからD-sub15ピンへの変換コネクタが必要な場合には，各自でご用意ください．D-sub15ピン以外では接続できませんのでご了承ください．
7．電源ケーブルを必ずご持参ください．バッテリーでのご使用はトラブルの原因となります．

８．発表時間は討論を含め一題20分です．

**■各種会議日程**

理 事 会：10月23日（木）　12時～　　慶應義塾大学医学部 北里記念図書館２階　第１会議室
評議員会：10月23日（木）　12時30分～　慶應義塾大学医学部 北里記念図書館２階　北里講堂
総　　会：10月23日（木）　16時30分～　慶應義塾大学医学部 北里記念図書館２階　北里講堂

**■大会事務局**

〒160-8582　東京都新宿区信濃町35
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学会のホームページにて，最新のお知らせ，注意事項，プログラムなどを掲載していきます．併せてご確認，ご利用ください．
学会ホームページ　http://www.blood-sub.jp/
6th CIBSR ホームページ（英語） http://www.convention.co.jp/b-sub2008

# 第15回年次大会 及び 第6回CIBSR 日程表

## 10月23日（木） October 23
北里講堂

| | | |
|---|---|---|
| 9:00 | | |
| 9:50 | 開会の辞 | |
| 10:00 | シンポジウム 「輸血代替としての利用法」 | |
| 10:30 | | |
| 11:00 | | |
| 11:30 | | |
| 12:00 | 昼休み | 理事会 12:00～第一会議室 |
| 12:30 | | 評議員会 12:30～北里講堂 |
| 13:00 | | |
| 13:20 | 会長講演（第15回年次大会） 堀之内宏久（慶應義塾大学） | |
| 14:00 | 一般口演 | |
| 14:30 | | |
| 15:00 | 休憩 | |
| 15:10 | | |
| 15:30 | 「機能性医薬」 | |
| 16:00 | | |
| 16:30 | 総会 | |
| 17:00 | 閉会の辞 | |
| 17:10 | | |
| 17:30 | 懇親会 レストランオアシス（慶應義塾大学病院 新棟11階） | 第6回CIBSR参加受付（新棟11階 大会議室前） |
| 18:00 | | |
| 18:30 | | |
| 19:00 | | |
| 19:30 | | |

## 10月24日（金） October 24
新棟 New Hospital Wing (Shinto) 11F

| | | |
|---|---|---|
| 8:50 | Opening Remarks | |
| 9:00 | Plenary Lecture I | Makoto Suematsu (Keio University) |
| 9:40 | Keynote Lecture I | Marcos Intaglietta (Univ. of California, San Diego) |
| 10:20 | Coffee Break | |
| 10:30 | Educational Lecture | Hirosuke Kobayashi (Kitasato University) |
| 11:10 | New Material | |
| 11:50 | | |
| 12:10 | Lunch Time Symposium | |
| 13:10 | Presidential Address (6th CIBSR) | Koichi Kobayashi (Keio University) |
| 13:40 | Symposium I PART I | *Cutting Edge of Research of Artificial Oxygen Carriers* |
| 14:00 | | |
| 14:30 | | |
| 15:00 | Coffee Break | |
| 15:10 | | |
| 15:30 | Symposium I PART II | *Cutting Edge of Research of Artificial Oxygen Carriers* |
| 16:00 | | |
| 16:30 | | |
| 17:10 | | |
| 17:30 | | |
| 18:00 | | |
| 18:30 | Banquet | Tokyo Dome Hotel |
| 19:00 | | |
| 19:30 | | |

## 10月25日（土） October 25
新棟 New Hospital Wing (Shinto) 11F

| | | |
|---|---|---|
| 8:30 | | |
| 9:10 | Plenary Lecture II | Warren M. Zapol (Massachusetts General Hospital) |
| 9:50 | Keynote Lecture II | A. Gerson Greenburg (Biopure Corporation) |
| 10:00 | Coffee Break | |
| 10:30 | Symposium II *Acceptable Vascular Reaction in HBOC* | |
| 11:00 | | |
| 11:20 | Invited Lecture | Jonathan S. Jahr (Univ. of California, Los Angeles) |
| 12:00 | Lunch | |
| 12:30 | | |
| 13:00 | Session *Gas Carrier and Gas Biology* | |
| 13:30 | | |
| 14:00 | | |
| 14:30 | | |
| 15:00 | Closing Remarks | |
| 15:15 | | |
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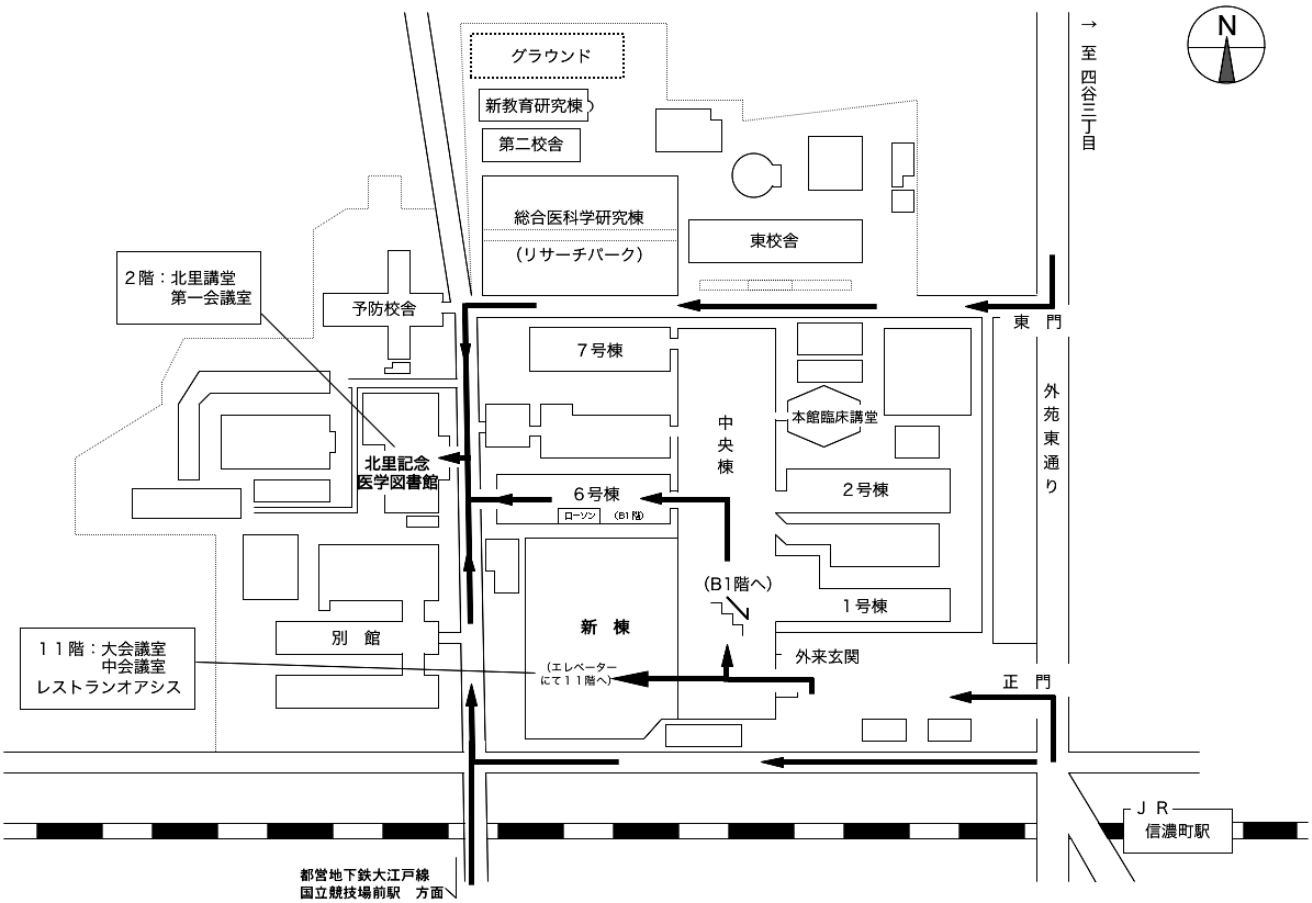
# 交通案内図




■JR総武線　「信濃町」駅下車 徒歩約５分

■都営地下鉄大江戸線「国立競技場」駅下車（A１番出口），徒歩約５分

## 10月23日（木）　October 23（THU）

**9：50〜10：00　開会の辞**

**10：00〜12：00　シンポジウム「輸血代替としての利用法」**

座長：高折 益彦（東宝塚さとう病院）

1. Beagle犬を用いた40%脱血ショックにおけるHb小胞体の蘇生効果および中長期生存後の安全性の検討
   池田 達彦（慶應義塾大学医学部呼吸器外科）
2. 腎傷害による制御不能出血モデルに対するヘモグロビン小胞体の蘇生効果
   勢司 泰久（慶應義塾大学医学部呼吸器外科）
3. 血管損傷による制御不能出血性ショックの蘇生―人工酸素運搬体の効果について―
   堀之内 宏久（慶應義塾大学医学部呼吸器外科）
4. マウス肺切除＋周術期出血モデルでのヘモグロビン小胞体投与の有効性の検討
   河野 光智（慶應義塾大学医学部呼吸器外科）
5. 人工酸素運搬体,ヘモグロビン小胞体（HbV） の in vitro におけるヒト造血幹/前駆細胞への影響
   藤原 満博（北海道赤十字血液センター）
6. カプセル型人工酸素運搬体の臨床試験実施に向けた課題
   金田 伸一（テルモ株式会社研究開発センター）

**12：00〜13：20　昼食**　　**12：00〜12：30　理事会**

**12：30〜13：00　評議員会**

**13：20〜14：00　会長講演**

座長：小林 紘一（慶應義塾大学）

ガスキャリアとしての人工酸素運搬体と肺循環
堀之内 宏久（慶應義塾大学医学部呼吸器外科）

**14：00〜15：00　一般口演**

座長：東　寛（北海道赤十字血液センター）

1. ラット摘出潅流心臓での虚血―再潅流障害に対するヘモグロビン小胞体の保護効果―第3報
   中島 淳（防衛医科大学校内科1）
2. 出血性ショックモデルラットにおける頻回投与時のヘモグロビン小胞体の体内動態特性評価
   田口 和明（熊本大学大学院医学薬学研究部）
3. ヘモグロビン小胞体（HbV）が免疫系に及ぼす影響
   高橋 大輔（北海道赤十字血液センター）

**15：00〜15：10　休憩**

**15：10〜16：30「機能性医薬」**

座長：武岡 真司（早稲田大学大学院先進理工学研究科）

1. 酸素親和性の異なるカプセル型人工酸素運搬体の作用
   金田 伸一（テルモ株式会社研究開発センター）
2. ヘモグロビン小胞体の表面荷電基の特徴
   宗 慶太郎（早稲田大学理工学術院理工学研究所）

3. アルブミンーフラーレン錯体の光物性と細胞毒性
小松 晃之（早稲田大学理工学術院理工学研究所）

4. 血小板凝集をトリガーとしてADPを放出するリポソームの血小板代替物としての止血能評価
岡村 陽介（早稲田大学大学院先進理工学研究科生命医科学専攻）

**16：30〜17：00　総会**

**17：00〜17：10　閉会の辞**

**17：30〜19：30　懇親会（新棟11階　レストランオアシス）**

# 会長講演

## ガスキャリアとしての人工酸素運搬体と肺循環

堀之内 宏久
慶應義塾大学医学部呼吸器外科

呼吸器外科領域では治療手段として通常，肺切除を行っている．肺葉切除が現在の治療の主流であるが，状態が許せば一側肺全摘を行うこともある．肺切除後のガス交換，循環状態がどのように変化するかは治療に直結する重要な検討課題である．

肺切除が行われると肺血管床が減少し，肺動脈圧が上昇していることが予想される．肺切除の周術期にSwan-Ganzカテーテルを肺動脈内に留置して肺切除後の肺循環の変化について検討を行うと，術後に心拍出量は増大するが，肺動脈圧に予想されたような上昇は認められなかった．以上のことから，肺循環における肺動脈圧を規定する因子は肺血管床のみではないことが明らかである．また，周術期に急性肺傷害を呈した患者では肺動脈圧の上昇が認められていた．血管床の減少のみではなく，肺循環のコンプライアンスが減少していることが示唆された．このような肺循環を調節する因子を明らかにすることが肺切除を安全に行うために必要と考えられる．

肺動脈圧を調節する因子は，数多くあるが，生理活性ガスが調節因子として深く関わっていることが知られている．酸素はガス交換の主役であるが，肺動脈のトーヌスを調節する重要な役割を果たしている．低酸素環境下で肺動脈圧が上昇する現象が，いわゆるHypoxic pulmonary hypertensionであり，現在我々はこの性質を利用して，片側肺を無気肺とし，無気肺となった肺のHypoxic vasoconstrictionを利用してVQ不均等分布を意図的に作成，ガス交換を安定させて安全に肺切除を行っている．この反応は可逆的である．また，高濃度の酸素を長期に使用すると，Hyperoxia induced lung injuryを引き起こし，不可逆性の間質性変化を惹起することが知られている．RatのHyperoxia lung injuryモデルを用いてサイトカインの影響を調べたところ，高濃度酸素暴露により，TNF$\alpha$，PDGFが組織内で活性化していることが明らかとなり，酸素そのものがガス交換局所で高い生理活性をもち，この生理活性により肺循環に変動が起こることが証明されている．もう一つの重要な生理活性ガスが，一酸化窒素NOである．NOは血中で半減期が短く，局所の血流を調節する役割を担っている．肺循環には吸入により肺動脈の拡張が認められ，肺傷害に陥った肺の肺動脈圧を低下させる薬剤として使用されている．

一方人工酸素運搬体は体内で酸素を運搬するために開発されたが，修飾ヘモグロビンを用いた人工酸素運搬体では，投与により血圧，肺動脈圧が上昇することが明らかとなった．これは，人工酸素運搬体がNOを吸着するためであると信じられてきた．しかし，NOを吸入させることで，血圧の上昇を制御できる可能性が示唆され，血管収縮には$O_2$,$N_2$以外の要素があることが考えられる．

我々が開発中のヘモグロビン小胞体では，Beagleを用いた50％脱血蘇生試験で5分間にわたる一過性の肺動脈の有意な上昇が観察された．投与直後の可逆性の変化は微粒子系であり，血管外への漏出がほとんどないためと考えられた．また，この一過性の肺動脈の上昇はインドメサシンの前投与では抑制できなかった．Hb小胞体の持つ一過性の肺動脈上昇効果はHbの持つ本来の一酸化窒素消去現象である可能性がある．

一酸化炭素COも生体内では非常に強力な生理活性ガスであり，ヘムオキシゲナーゼにより産生されるが，現在まで定量的な投与法がなかったために，吸入による毒性のみが強調されてきた．人工酸素運搬体にCOを吸着させて投与することにより，定量的な投与が可能となり，種々の病態の治療に応用できる可能性がある．酒井らは血管収縮を制御して，臓器灌流を確保しながらショックの蘇生を行う蘇生液として，また，COを放出した後は酸素運搬体として臓器の酸素代謝を維持する機能を発揮して臓器機能を保全して蘇生効率を上げる酸素運搬体として機能することを発表している．

今後，人工酸素運搬体の研究を通してガス運搬体の機能解明が進むと，赤血球代替としてのみでなく，新たな酸素治療，生理活性ガス治療が開発されることになると考えられ，人工酸素運搬体開発の大きな一つの柱となるものと思われる．

# シンポジウム-1

## Beagle犬を用いた40％脱血ショックにおけるHb小胞体の蘇生効果および中長期生存後の安全性の検討

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＜目的＞Hb小胞体（HbV）の臨床応用を考えた場合，その安全性の評価は必須である．これまでに生体投与に関する有効性と安全性について様々な角度より検討がなされてきた．以前よりビーグル犬を用いて50％出血ショックモデルを作成し，HbVはアルブミン生食，脱血血液と同様の蘇生効果があることが示された．今回は中長期の安全性の評価のため，40％出血モデルを作成し，HbV，または脱血血液を用いて蘇生し，その後生存させることとした．
＜方法＞月齢6ヶ月のビーグル犬16頭を使用した．全身麻酔を行った後に，右大腿動脈に動脈圧モニター用，および脱血用のカテーテルを挿入した．左前肢に静脈ラインをとり，蘇生液の投与経路とした．計測のための機器を装着し，状態が安定化した後に体重から求めた循環血液量の40％相当量を脱血した．脱血に従い血圧の低下を認め，60分間にわたり平均血圧が50mmHgを上回らないように維持した．その後，脱血血液（Autologous shed blood　ASB群），Hb小胞体分散液（HbVを5％アルブミン生食に分散した液体．Hb濃度は8.6 g/dl：HbV群）を用いて蘇生を行った．蘇生液投与後4時間，全身麻酔下に循環動態を確認した後に半覚醒の状態とし，自室へ戻した．以後1日目，3日目，7日目，14日目，28日目，56日目，84日目，168日目，365日目に体重，CBC，血液生化学を測定した．また28日目，168日目または365日目に犠牲死させ病理組織学的所見を検討した．
＜結果と考察＞ASB群で7頭，HbV群で9頭使用した．28日，168日，365日でASB群は2頭，2頭，3頭犠牲死させ，HbV群は3頭ずつ犠牲死させた．全例とも生存し，HbV群はASB群と同様の蘇生効果を示した．体重変化は2群とも同様に増加し，365日後には12kgに達した．Hct値はHbV群で蘇生後低下したが14日でASB群と同等な値に上昇した．WBC，PLTは2群ともにほぼ同様の変化を示した．AST，ALT，CPKが1日目に両群とも上昇したが3日目にはbaselineに復した．ショックの影響と考えられた．T-Cholは3，7日目にHbV群で上昇したが14日目以後は2群とも同様な経過を示した．Lipaseは7，14日目にHbV群で低下したが28日目以後は2群とも同様な経過を示した．病理組織学的評価ではHbV群で28日目に犠牲死させた3頭で肝臓，脾臓において少量の褐色色素沈着を認めた．HbVの代謝過程での変化と考えられた．その他，心臓，肺，腎臓，膵臓，小腸，大腸，食道，精巣，胸腺，副腎において明らかな異常を認めなかった．
＜結論＞中動物での脱血ショック蘇生モデルを用いた中長期生存の実験での蘇生効果および安全性を確認した．

# シンポジウム-2

## 腎傷害による制御不能出血モデルに対するヘモグロビン小胞体の蘇生効果

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【目的】大量出血患者の搬送に際して欧米では積極的な輸液蘇生が行われており，血液希釈を生じる．一方，本邦では輸液は通常行われておらず，血液循環量維持の点で不利である．そこで直径約250nmの細胞型人工酸素運搬体であるヘモグロビン小胞体（HbV）を輸液に用いることで，酸素供給と循環量維持を両立し，生存性向上が図れると考え，実験を行った．
【方法】体重約350gのWistar系雄性ラットをセボフルラン吸入麻酔下で維持し，血圧測定，輸液及び採血用にカテーテルを挿管した．また心電図，末梢組織に酸素電極及びレーザードップラー血流計を設置した．更にHESを用いて血液交換し，ヘマトクリット値を約1/3に低下させた後，左腎臓へ経皮的に針金を刺入して引き絞り，臓器裂傷を作製した．その後，平均動脈圧（mAP）が30mmHgに低下した時点から速度56mL/kg/hrで輸液した．輸液中にmAPが40mmHg以上に上昇した場合は輸液を停止し，35mmHg以下に低下した場合は再開して血圧を制御した．群構成はHbVを5％リコンビナントアルブミン（rHSA）に分散した被験薬群（HbV群）及び媒体である5％rHSAの代用血漿群，無輸液の無蘇生群の計3群とした．観察はmAP=30mmHg時点から最大60minまで実施し，経時的に心拍数，mAP，呼吸数，酸素分圧，血流量，血中乳酸値及びpHを測定した．更に生存時間から1hr生存率，総出血量及び総輸液量から見かけの循環血液量変化率を算出した．
【結果】HbV群は生存性が最も高く，死亡を認めなかったが，他群では途中止死亡例が出現した．見かけの循環血液量は各群とも減少を示したが，HbV群は比較的小幅であった．更にHbV群は，心拍数が序盤で低下したものの10min時点から安定，mAPが序盤で上昇した後，他群よりも高値，呼吸数が序盤で低下したものの10min時点から安定に推移した．末梢酸素分圧は低下傾向を示したものの10min時点からは平均値で約15mmHgに安定し，末梢血流は輸液中にほとんど変動しなかった．血中乳酸値は序盤で上昇し，以後は極僅な上昇傾向に転じたが，他群に比較して抑制傾向であった．pHは出血直前より極僅な低下傾向に推移した．これらの結果は他群に比較して同等以上の良好な成績であった．
【考察】制御不能出血に対して人工酸素運搬体を用いた蘇生を試みたところ，無蘇生や代用血漿輸液と比較して，生存時間の延長など生存性向上効果が認められた．一方，末梢組織での酸素分圧は期待される程の上昇ではなく末梢血流上昇も充分ではなかった．しかし乳酸値及びpHの推移から，HbVを用いた蘇生は酸素代謝の改善に寄与したことが示されており，赤血球よりも粒子径の小さいHbVが低血流量下の末梢でも酸素を運搬すると推察された．

# シンポジウム-3

## 血管損傷による制御不能出血性ショックの蘇生
## ―人工酸素運搬体の効果について―

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出血性ショックの治療は出血を制御し，減少した循環血液量を補てんし，臓器機能を回復させることにある．受傷現場で出血のコントロールがつかない場合に治療施設まで，輸液を行いながら搬送することが通常行われている．輸液製剤と輸液速度については現在まで，いろいろな検討が行われてきた．今回，血管損傷による制御不能出血性ショックモデルにおけるヘモグロビン小胞体（HbV）の蘇生効果を検討した．

【方法】雄性Wistarラット（350g）を用い，開腹し，腹部大動脈を剥離露出，プラスチック装具にて腸を圧排し，腹背方向に24G針を貫通させ，出血を惹起した． 1分以内に平均血圧が20mmHg以下となった動物で実験を継続した．20mmHg以下となった時点で輸液を開始し，規定量の輸液が終了した後2時間にわたってMAP，HR，呼吸数，Ht，白血球数，Hb，乳酸，ピルビン酸を測定した．無蘇生群，および蘇生液として出血量の3倍量の生食，等量の同種血液，アルブミン生食，HbVを用いて検討した．

【結果】出血量は無蘇生群8.0 ± 0.9ml，生食群14.3 ± 2.9ml，同種血輸血群12.6 ± 4.1ml，5％rHSA群11.8 ± 4.0ml，HbV群9.8 ± 3.7mlであった．生食群では出血量も多く，血圧を上昇させて維持することはできなかったが，他の群では生食群に比して出血率の減少を認めた．HbV群では出血量の減少，生存時間の延長，乳酸・ピルビン酸比の変化率の減少を認めた．

【考察】制御不能の出血性ショックの状態に対する蘇生では，大量の晶質液のみでは生存時間を延長することはできず，膠質液が生存時間の延長に有効であると思われた．臓器での嫌気解糖の程度を検討する目的で乳酸・ピルビン酸比を測定したが，血液による蘇生では，乳酸ピルビン酸比の上昇を認めず，HbVによる蘇生では，乳酸ピルビン酸比は上昇を認めたが，出血量，生存時間の延長を認めた．HbVによる蘇生は生存を延長し，臓器の酸素代謝を改善する可能性があると考えられた．

# シンポジウム-4

## マウス肺切除＋周術期出血モデルでのヘモグロビン小胞体投与の有効性の検討

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輸血の代用として開発されたヘモグロビン小胞体は，ヘモグロビンを期限切れのヒト赤血球から精製濃縮し，リン脂質小胞体に内包した小球粒子（直径：250nm）である．血液型がなく，ウィルスなどの感染源を排除，室温で2年以上の備蓄が可能であり，これまでに酸素運搬体として機能することが確認され，出血性ショック蘇生液としての効果と安全性が動物実験において実証されている．臨床応用を視野に入れた多くの研究が進行中であるが，本研究では輸血の頻度が高い待機手術への応用を動物モデルにおいて検討した．マウスに左肺切除と循環血液量の25%，或いは40％の交換輸液を行う，周術期出血を想定した動物モデルを新たに作成した．C57BL/6マウス，雄性，8～10週齢（20～22g）を用いて人工呼吸器管理下に左肺全摘術を行った．25％交換輸液は尾端を切断して出血を起こさせた後，尾静脈から異なる輸液を行った．40%交換輸液は頚動脈にカテーテルを挿入し，0.1mlの脱血と同量の試料溶液投与を6回繰り返して循環血液量の40％を交換した．このモデルを用い，乳酸リンゲル液，5%アルブミン生食液，マウス保存血液，そしてヘモグロビン小胞体分散液を輸液し，生存率，体重減少，摂食量，自発運動量の変化を観察した．回復の過程を比較することで，ヘモグロビン小胞体の有効性と安全性を検討した．25%の交換輸液実験では，左肺全摘術後，いずれの群においてもマウスは長期生存可能であった．ヘモグロビン小胞体の投与により，術後早期の体重減少は乳酸リンゲル液投与と比較して抑制される傾向にあった．また，摂食量はいづれの群においても術直後に低下するが，7日目まで群間で有意差を認めず，回復した．術後に低下した自発運動量も群間に優位差を認めずに回復した．また，40％の交換輸液実験では，肺全摘術後，ヘモグロビン小胞体投与によりマウス保存血輸血と同等の生存率が得られた．術後の体重減少および回復，食事摂取量の回復もヘモグロビン小胞体投与後とマウス保存血投与後とでは有意差は認めなかった．左肺全摘による呼吸機能低下，酸素化能低下状態でヘモグロビン小胞体が有効に機能したと考えられた．体重変化と摂食量による今回の検討では，外科的侵襲からの回復過程に深刻な影響を与えなかった．これらの結果から，ヘモグロビン小胞体は，外科手術による出血に対しても安全に使用できる可能性が示唆された．

# シンポジウム-5

## 人工酸素運搬体，ヘモグロビン小胞体（HbV）のin vitroにおけるヒト造血幹/前駆細胞への影響

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【目的】HbVは，動物モデルにおいてその有効性が示されている．非臨床試験がおこなわれるうえで，HbVのヒト血液細胞への適合性を検討する必要がある．HbVは投与後，主に肝臓，脾臓のほか，骨髄にも分布することから，骨髄における造血機能に対し少なからず影響するのではないかと考えられた．そこでヒト造血前駆細胞の増殖・分化および造血幹/前駆細胞の増幅に対する影響について，in vitroの培養系において評価をおこなった．
【方法】（1）造血前駆細胞の増殖・分化の評価：ヒト臍帯血単核球より純化したCD34陽性細胞を，異なる濃度（3%(v/v)まで）のHbVを添加したイスコフ改変ダルベッコ培地（+ 30% FBS, IL-3, SCF）中で，10日間の液体培養をおこなった．赤芽球系の細胞（CD235a陽性細胞）へ分化させるためにはerythropoietinを，顆粒系の細胞（CD15陽性細胞）へ分化させるためにはGM-CSF + G-CSFをそれぞれ上記培地に添加した．HbVとのインキュベーション期間の影響をみる場合は，HbV添加の上記培地にてCD34陽性細胞を20時間および3日間培養し，HbVを除去後，10日目まで培養した．(2) CD34陽性細胞の増幅の評価：confluentにしたヒト骨髄ストローマ細胞株にCD34陽性細胞を浮遊させ，HbVを添加した無血清培地X-VIVO 10（+造血支持サイトカイン）にて共培養した．1週間後に等量の培地を加え，2週間後に全細胞数およびCD34陽性細胞をカウントした． HbVとのインキュベーション期間の影響を見る場合は，HbV存在下でCD34陽性細胞とストローマ細胞株を3日間または7日間共培養後，HbVを除去し，2週間目まで共培養をおこなった．
【結果】（1）CD34陽性細胞の液体培養にて，HbVが10日間継続してある場合は，HbVの濃度に依存してCD235a陽性細胞およびCD15陽性細胞の増殖抑制がみられた．また，分化した赤芽球系の細胞（CD235a+CD45-）および顆粒球系の細胞（CD15+CD33-）の割合も減少した．一方，20時間および3日間のHbVとのインキュベーションの場合には，CD235a陽性細胞およびCD15陽性細胞の増殖抑制はみられなかった．(2) ヒト骨髄ストローマ細胞株との共培養系において，HbVが継続してある場合には，HbVの濃度に依存して全細胞数およびCD34陽性細胞の増幅の抑制がみられた． HbVに7日間曝した場合は14日間と同様な抑制が見られたが，3日間曝した場合の全細胞数およびCD34陽性細胞増幅の抑制は軽微であった．
【考察】HbVに曝される期間に依存して，ヒト造血前駆細胞の増殖・分化の抑制ならびにヒト造血幹/前駆細胞の増幅の抑制がみられた．In vivoでは，HbVは骨髄マクロファージ内に投与後1-3日に蓄積し，7日では激減する．さらにHbVとヒト造血幹/前駆細胞と直接相互作用することは少ないと考えられる．よって，HbVとヒト造血幹/前駆細胞が直接相互作用するin vitroの閉鎖系において，3日間ならば抑制効果が少ないという結果は，in vivoにおいてHbVの造血への影響は少ないことを示唆すると考えられる．

# シンポジウム-6

## カプセル型人工酸素運搬体の臨床試験実施に向けた課題

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輸血用血液製剤の代替物，あるいは酸素治療用製剤として，人工酸素運搬体の医療への応用が待たれて久しいが，未だ実用化に向けた開発の途上にある．最近，FDA主催のワークショップにてヘモグロビン（Hb）利用非カプセル型人工酸素運搬体について，その問題点が改めて指摘され，人工酸素運搬体の開発に大きな課題が突きつけられたと言える．一方，カプセル型人工酸素運搬体においても，これまで以上に，Hbをカプセル化することの有用性，リポソーム製剤としての特性，安全性について，多くの情報を求められることが予測される．我々は，ヒト赤血球由来のHbをリポソーム内に封入したカプセル型人工酸素運搬体（LEH）の開発を行って来たが，本発表では，前述の課題への対応も含め，LEHの開発の進め方について議論したい．

品質保証の観点からは，LEHではリポソーム製剤の特性から，最終滅菌に課題が残り，無菌操作製剤として治験薬GMPに準拠した製造が可能な工程を設定している．また，Hbはヒト血液に由来するものであり，LEHでは，医薬品であるリポソーム製剤と，生物由来製品に求められる管理が共に必要とされると考えており，夫々に，対応すべき規制事項も踏まえた，試験評価，バリデーション等を行っていく必要がある．

また，人工酸素運搬体の酸素運搬能力については，理論的には，Hb濃度，酸素親和性と動脈，末梢組織間での酸素分圧格差に依存するが，必要とされるHb濃度，酸素運搬量や酸素親和性の最適域は，臨床適用の領域，使用条件により変る可能性もあり，議論の余地があると考えている．また，リポソーム製剤については，近年のDDS製剤開発の過程で，その有用性と課題に関する情報が蓄積されてきている．LEH開発に際しては，前述のHbをカプセル化する意義の検証に加え，これらの情報も活用し，有効性や安全性の評価を進める必要がある．特に，リポソーム製剤に対する生体反応には種差が存在することを経験しており，複数の動物種を用いた評価による，臨床的なリスクの検討を進めている．さらに，血液代替物の場合，他のリポソーム製剤とはけた違いの大量投与や，高度の出血状態等，生体の恒常性が大きく損なわれた条件下での投与が想定され，また，酸素治療用製剤としても，虚血病変への影響等，非臨床試験に関しては，本製剤の使用条件の特殊性を考慮した試験設計に留意することが必要と考える．

また，臨床応用に向けては，各種の臨床検査指標に対し，LEHは，その製剤の特性故に影響を与えることが確認されており，その対策を検討すると共に，特に血液代替物としては，臨床試験における代替エンドポイントの設定が重要な課題であると認識している．

以上のように，LEHの臨床応用に向けては，多くの課題があるが，従来に無い，新しい特徴を持った医薬品であることから，今後，早い段階から，規制当局との対話を持ちつつ，必要な要件に関し対応を行うことで，臨床試験開始に向け進んでいきたいと考えている．

# 一般口演-1

## ラット摘出潅流心臓での虚血-再潅流障害に対するヘモグロビン小胞体の保護効果－第3報

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【目的】我々は，ラットの摘出心臓をランゲンドルフ潅流する方法を用いて，ヘモグロビン小胞体（HbV）が虚血-再潅流時の心機能を有意に回復させることを明らかにしてきた．本研究では，Hb小胞体の心機能回復効果のメカニズムを解明する一環として，この効果がmitochondria $K_{ATP}$-channel活性を経由する否かを検討するため，このchannel活性を選択的に抑制する5-hydroxydecanoate（5-HD）を用いて以下の実験を行った．虚血-再潅流後の心機能を回復させる比較対照として，mitochondria $K_{ATP}$-channel openerのdiazoxide（Dia）とischemic preconditioning（IPC）を用いた．
【方法】9-12週齢のWistar系雄性ラットにヘパリン1000 Uを腹腔内投与し，nembutal 60 mg/Kgを腹腔内投与して麻酔した．心臓を取り出し，Krebs-Henseleit buffer（KH-buffer）を用いて静水圧100 $cmH_2O$，37℃でランゲンドルフ潅流した．左心室にラテックスバルーンを挿入し，圧トランスデューサーを介して，心機能を連続的に記録した．HbVは，Hb濃度が0.33 g/dLになるようにKH-bufferで希釈・懸濁し，Diaと5-HDはどちらも100 $\mu$MでKH-buffer に溶解させ，37℃に加温して95% $O_2$ + 5% $CO_2$混合ガスを通気した．約20分間KH-bufferでcontrol潅流を行った後，対照群，HbV群，5-HD + HbV群，Dia群，5-HD + Dia群，IPC群，5-HD + IPC群の7群（各群n = 3）に分けて以下の実験を行った．対照群では，control潅流の後，虚血25分-再潅流30分の処置を施した．HbV群では，虚血25分-再潅流30分処置の前に，その希釈・懸濁液を10分間潅流し，Dia群ではその溶液を15分間潅流した．IPC群では，虚血25分-再潅流30分処置の前に，虚血5分-再潅流5分を3回繰り返した．5-HD溶液は，HbV懸濁液あるいはDia溶液の潅流およびIPC処置の10-15分前から潅流を開始し，虚血25分-再潅流30分処置の前まで継続した．
【結果】① 対照群では，全例で再潅流後の左室発生圧の回復は認められなかった．② Dia群とIPC群では，再潅流開始後には全例で左室発生圧の回復が見られ，その程度は虚血開始前値の60%を越えた．③ 5-HD 100 $\mu$Mを潅流することで，Diaと IPCの心機能回復効果は完全に抑制された．④ HbV群でも，全例で再潅流後の左室発生圧の回復が認められたが，その効果は弱かった．⑤ HbVによる心機能回復効果は，5-HDで抑制されなかった．
【考察】これらの実験結果は，HbVの虚血-再潅流後の心機能回復効果がmitochondria $K_{ATP}$-channel活性を介さない可能性があることを示唆した．

# 一般口演-2

## 出血性ショックモデルラットにおける頻回投与時のヘモグロビン小胞体の体内動態特性評価

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【緒言】
ヘモグロビン小胞体（HbV）はリン脂質二重膜にヒト赤血球由来ヘモグロビン（Hb）を高濃度に封入した細胞型人工酸素運搬体である．近年，同一動物にポリエチレングリコール（PEG）修飾リポソーム製剤を，1回目投与後にある投与間隔で2回目投与を行うと2回目投与されたリポソームの血中滞留性が1回目投与時の血中滞留性より著しく低下するAccelerated Blood Clearance 現象（ABC現象）が報告されている．HbVの臨床適用は手術時や大量出血時であり，頻回投与が予想される．そのため，PEG修飾リポソーム製剤であるHbVにおいても，ABC現象が誘導される可能性は十分に考えられ，HbVの頻回投与時の体内動態の把握はHbVを上市する上で重要である．このような背景の下，本研究は，HbVの内部Hbをヨウ素（$^{125}$I）標識した$^{125}$I-HbVを用い，40％出血性ショックモデルラットにおける頻回投与時と単回投与時の体内動態を比較検討した．
【方法】
実験動物はSD系雄性ラット（6週齢，180～200g）を用いた．出血性ショックモデルラットの作製は血圧を30分間，40mmHg以下に保ちながら，全血液量の40％を脱血することで出血性ショックモデルを作製した．HbV投与液は，IODOGENを用い$^{125}$I-HbVを調製した．体内動態の検討は，出血性ショックモデルラットの作成を行い，非標識HbV投与液（1400mg/kg）で蘇生し，1時間後に$^{125}$I-HbV（1400mg/kg）を再び投与し，経時的に開腹，採血及び肝臓・脾臓を摘出した．血液及び各臓器の放射活性はオートウェルガンマーカウンターにより測定した．各速度論パラメータはMULTIを用い算出した．また，HbV特異的IgG及びIgMの検出はELISA法により評価した．
【結果及び考察】
出血性ショックモデルラットにおける2回目投与時の消失半減期は単回投与時に比べ延長しており，その値は健常時と同レベルまで回復した．加えて，肝臓・脾臓への分布は，投与初期では単回投与時に比べて分布量が少ないものの，投与後12時間を過ぎると初回投与に比べ臓器分布の増大と排泄の遅延が認められた．以上，本研究の条件では出血性ショック時においてHbVを頻回投与してもABC現象は誘導されないことが確認され，HbVの臨床使用に向けた有用な基礎データを提示することができた．

# 一般口演-3

## ヘモグロビン小胞体（HbV）が免疫系に及ぼす影響

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【目的】ヘモグロビン小胞体（HbV）は，高純度濃厚ヒトヘモグロビン溶液を脂質二分子膜で被覆し，表面をポリエチレングリコールで修飾した人工酸素運搬体である．我々はこれまで，HbV投与後のラット脾細胞中に，正常ラット脾細胞のConA刺激による細胞増殖を一過性に抑制させる細胞が含まれることを見出してきた．今回は，細胞増殖の抑制に関与する細胞の特性を明らかにする目的でMHC classII抗原の発現に着目して検討を行った．

【方法】WKAHラットに20%相当のHbV，対照として生理食塩水をエーテル麻酔下，尾静脈より輸注し，16時間後に脾臓の摘出を行った．摘出した脾臓から脾細胞浮遊液を調整し，ラットMHC classII抗体（OX6）結合磁性ビーズを用い，MHC classII陽性および陰性画分を分画した．それぞれの画分についてサイトスピンを作成し，May-Grunwald Giemsa染色を行い，顕微鏡下でHbV貪食細胞の陽性率を算出した．また，それぞれの画分の増殖能に与える影響について評価を行うために，未処理ラット脾細胞にHbV輸注ラットから分画したMHC classII陽性および陰性画分を1:1, 1:0.2の割合となるように混合し，低濃度ConA刺激（0.3 μg/mL）による正常ラット脾細胞の増殖能を測定した．さらにリポソーム貪食細胞の細胞表面マーカーを調べるため，FITC標識空リポソームを輸注し，同様にMHC classII陽性および陰性画分を分画し，フローサイトメーターを用いてそれぞれの画分におけるFITC陽性細胞，すなわちリポソーム貪食細胞の細胞表面マーカーを調べた．

【結果・考察】HbV輸注ラット脾細胞中のclassII陽性および陰性細胞画分どちらにもHbV貪食細胞が含まれていた．両者の陽性細胞率はclassII陽性画分で4%，陰性画分で14%であり，HbV貪食細胞はclassII陰性画分に有意に多く存在していた（$p<0.01$）．また，細胞増殖への影響を確認した結果，未処理脾細胞：classII陽性（陰性）脾細胞の混合比1:1の条件ではclassII陽性，陰性いずれの画分でも増殖抑制を認めたが，1:0.2の条件ではClassII陰性画分でのみ増殖抑制を認めた．貪食細胞の細胞表面マーカーはいずれの画分もCD3-,CD4mid,CD8α-, CD11b bimodal, CD103-, CD43-であった．以上のことからHbV輸注後のラット脾細胞に含まれる細胞増殖抑制に関与する細胞集団はMHC classII分子の発現の有無により，2種類のサブセット分けられると考えられた．

# 機能性医薬-1

## 酸素親和性の異なるカプセル型人工酸素運搬体の作用

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【緒言】
カプセル型人工酸素運搬体は，ヒト赤血球由来のヘモグロビン（Hb）をリポソーム内に封入した製剤である．カプセル型人工酸素運搬体製剤の特徴として，内水相のアロステリック因子の量や，pH等の条件により酸素親和性を比較的容易に調節可能な点が上げられる．本発表では，酸素親和性の異なる製剤の作用について，有効性，安全性の観点から評価した結果を報告する．

【方法】
人工酸素運搬体製剤：高親和性製剤H-LHb（$P_{50}$：9-12torr），低親和性製剤TRM-645（$P_{50}$：48-54torr）を用いた．

出血モデル：雄性SDラットにおいて，Hespander™を用いた40mL/kgの脱血，同時投与による血液希釈を行い，更に20mL/kgの脱血後，同量のHespander™を補液し，次いで5, 10, 20mL/kgの製剤を投与し，投与後，30分，1時間後の血漿中乳酸値を測定した．

脳梗塞モデル：雄性SDラットにおいて，中大脳動脈をナイロン糸製栓子により2時間閉塞させ，一過性の脳虚血を招来した後，0.2，1，5mL/kgの製剤を虚血開始後30分に投与した．血流再開22時間後に，神経症状を観察し，脳を摘出して脳切片を作製，TTC染色し，画像解析にて計測した梗塞部面積より梗塞巣体積を算出した．

機能的Hb濃度：雄性SDラットに20mL/kgの用量にて製剤を投与し，投与後，5分，6，24時間後に採血を行い，血中ヒトHb濃度を，抗ヒトHb抗体を用いたELISAにより，Hbメト化率をシアンメトHb法により測定した．

血管収縮作用：雄性SDラット頚動脈を摘出，リング状標本を作製し，Krebs-Henseleit液中に懸垂し，95%$O_2$+5%$CO_2$の混合ガス通気下で，phenylephrine添加時血管収縮のAchによる弛緩反応に対する製剤添加の影響を評価した．

【結果】
ラット出血モデルにおける血漿中乳酸値上昇に対し，高親和性，低親和性の何れの製剤もほぼ同等の投与用量依存的な抑制効果を示した．一方，一過性脳虚血モデルでも，何れの製剤も，投与用量依存的な梗塞体積の抑制効果を示したが，高親和性製剤の方が低用量で有意な効果を示した．また，神経症状スコアでは何れの製剤でも投与用量に依存し低減される傾向が見られたが，統計学的な差は認めなかった．血中でのHbの動態，メト化の進行速度には製剤間での統計学的な差は見られなかった．さらに，摘出血管の収縮性についても，製剤間で顕著な差は見られなかった．

【結論】
酸素親和性の異なる製剤間の比較において，局所の虚血に関しては高親和性の製剤がより効果的である可能性が示されたが，有効性，安全性の何れの点からも，著明な差は認められなかった．

# 機能性医薬-2

## ヘモグロビン小胞体の表面荷電基の特徴

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【緒言】ヘモグロビン（Hb）小胞体に関するこれまでの動物投与試験やヒト血液を対象とする*in vitro*混合試験では，補体系を含む免疫系の顕著な活性化を認めないことが確認されている．Hb小胞体の特徴的な表面物性の一つとして負電荷成分による電荷が挙げられる．本研究では，Hb小胞体に使用している負電荷成分による表面電荷の特徴を明らかにすると共に，血液適合性における表面荷電基の構造特性について知見を得ることを目的とした．

【方法】小胞体の電気泳動移動度をレーザードップラー法により測定し，ゼータ電位を算出した．また，小胞体表面の負電荷が媒介する静電相互作用のモデルとして，$Ca^{2+}$，カチオン性オリゴマー，カチオン性ポリマーを所定濃度で小胞体分散液に添加し，ゼータ電位の変動を測定した．雄性Wistarラット（体重250 ± 20 g）に対し，エーテル自発呼吸麻酔下，尾静脈に挿入した留置針を介して小胞体試料を投与した（投与量：5.6 mL/kg、投与速度：1mL/min）．投与後覚醒させ，1ないし24時間後に血液を採取し，遠心分離（$1 \times 10^3$ g, 10 min）により血球成分を除去．続いて超遠心分離（$3 \times 10^5$ g, 30 min）により小胞体を除去した．得られた試料について血清補体価を測定した．

【結果・考察】Hb小胞体に使用している負電荷脂質は中性付近では小胞体表面に比較的強い負電荷を与え,この電荷によりHbの内包効率が向上する．負電荷脂質含量の増大に伴いゼータ電位が負に増大して内包効率が向上するものの，-30mV以下でこの効果はほぼ一定となった．Hb小胞体の脂質組成では-40mV程度のゼータ電位を有していることから，負電荷脂質の含量が適正範囲に設定されていることを確認した．従来，負電荷リン脂質を含有する小胞体では，投与後の補体活性化や血小板減少などが報告され，表面電荷が補体活性化の主要因と考えられている．本検討では，Hb小胞体に使用している負電荷脂質は負電荷リン脂質と同程度の電荷を小胞体表面に与え，この電荷が関与する静電的相互作用も同等であることが示された．一方，投与後の血清補体価の低下や血小板減少は負電荷リン脂質を含有する小胞体のみで認められた．これらの結果は，小胞体表面の負電荷基の構造が生体反応における重要な因子であることを示唆する．

# 機能性医薬-3

## アルブミン－フラーレン錯体の光物性と細胞毒性

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小林 紘一[2]
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2 慶応義塾大学医学部呼吸器外科

ヒト血清アルブミン（HSA）は血漿蛋白質の約60wt％を占める補欠分子族を持たない単純蛋白質（分子量66,500）であり，血流中では膠質浸透圧維持のほか，疎水性薬物・代謝産物の運搬／貯蔵の役割を担っている．メトヘモグロビンから解離した鉄（III）プロトポルフィリンIX（ヘミン）も血中ではHSAに捕捉され肝臓へと運ばれる．我々はHSA-ヘミン錯体のX線結晶構造解析に成功し，ヘミンがHSAのサブドメインIBにチロシン161との軸配位により結合していることを見出した（1）．そこでヘモグロビン（Hb）のヘムポケット構造にならい，遺伝子組換え技術によりHSAのサブドメインIB内へヒスチジン（近位軸塩）を導入してみると，驚いたことに得られた組換えHSA-鉄（II）ポルフィリンIX（ヘム）錯体はHbのように酸素を吸脱着できるようになった（2, 3）．さらに配位酸素近傍のアミノ酸へ変異を加えると，rHSA-ヘム錯体の酸素親和性が調節できることもわかった（4）．他方，ヘムの中心金属を鉄から亜鉛に変換してみると，得られたHSA-Zn（II）ポルフィリン錯体は可視光照射により安定な励起三重項状態を形成することが可能で，白金コロイドを用いた水の還元反応（水素発生反応）の有効な光増感剤として作用することが明らかとなった（5）．このように我々は，血漿蛋白質であるHSAの多分子結合能を利用して，その内部に様々な機能性分子を結合させることにより，自然界には存在しないユニークな機能蛋白質を創製してきている．

今回はHSAにフラーレン誘導体を結合させたHSA-フラーレン錯体を合成し，その構造，光物性，一重項酸素生成能，さらには可視光照射下にける細胞毒性について検討した．トリス（ジカルボキシメチレン）［60］フラーレン（CF）はHSAに1:1（モル/モル）で結合し，安定なHSA-CF錯体を形成する．窒素下におけるレーザーフラッシュ照射後の励起三重項寿命は46$\mu$sで酸素濃度の増大に伴い減少したことから，酸素分子へのエネルギー移動により一重項酸素が生成しているものと考えられる（6）．Stern-Volmerプロットから決定した速度定数（$k_q$）は$2.2 \times 10^8$（$M^{-1}s^{-1}$），量子収率は0.46であった．腫瘍細胞（LY80）にHSA-CF錯体を添加し可視光照射すると，対照群（HSA-CF非添加群）に比べ顕著な細胞数の低下が認められた．HSA-CF錯体は光線力学治療（PDT）の新しい光増感剤として期待される．

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3. T. Komatsu, et al. *J. Am. Chem. Soc.*, 127, 15933（2005）.
4. T. Komatsu, et al. *J. Am. Chem. Soc.*, 129, 11286（2007）.
5. T. Komatsu, et al. *J. Am. Chem. Soc.*, 128, 16297（2006）.
6. Z. Qu, T. Komatsu, et al. *Bioconjugate Chem.*, 19, 1556（2008）.

# 機能性医薬-4

## 血小板凝集をトリガーとしてADPを放出するリポソームの血小板代替物としての止血能評価

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【目的】フィブリノーゲンγ鎖C末端ドデカペプチド（H12）を表面に結合させたリポソームは，活性化血小板間を架橋して血小板凝集を促進させる機能を持ち，血小板減少ラットに静脈投与すると出血時間を短縮させることを既に報告してきた．本報では，血小板凝集惹起剤（ADP）を内包させたH12結合リポソーム（H12-(ADP）リポソーム）を調製し，血小板代替物としての止血能増幅を評価した．

【方法】H12をリポソームに担持し，内水相にADP水溶液（1 mM, 血小板凝集試験では[8-$^{14}$C]ADPを使用）を内包した．H12-(ADP）リポソームをPRPに分散させ（[PLT] = 2.0 x $10^5$/μL，[小胞体] = f.c. 0.1 mg/mL），ADP刺激（[ADP] = f.c. 3 μM）にて血小板凝集を惹起させ，液体シンチレーションカウンターにてADP放出率を測定した．血小板減少症ウサギ（11週齢，2.5 kg）にH12-(ADP）リポソームを投与し，投与9時間までの出血時間を経時的に測定した．

【結果･考察】H12-(ADP）リポソーム［1］と（ADP）リポソーム［2］の血小板凝集能の比較では，［1］の透過度が約10％増大した．そこで，RI標識体を用いて同条件にて試験したところ，［1］は血小板凝集塊に有意に取込まれ，ADPの放出率は55.8 + 2.1 %と算出されたのに対し，［2］は全く血小板凝集塊に取込まれなかった．これは，［1］がH12を介して血小板と多点結合して血小板凝集に巻込まれ，その際に受ける物理的な刺激によって内包ADPが放出されたためと考えられる．

血小板減少症ウサギにH12-(ADP）リポソームをそれぞれ10, 20 mg/kgで投与したところ，出血時間はそれぞれ881 ± 303, 442+45秒であり，生理食塩水群（1696 ± 197秒）あるいは（ADP）リポソーム群（10，20 mg/kg で1503+481，1375+663秒）と比較して，投与量依存的に有意に出血時間を短縮させ，血小板輸血群のそれに匹敵した．この効果は投与6時間後まで持続でき，H12-(ADP）リポソームの有効血中濃度は100 μg/mL以上であると算出された．従って，ADP内包によってH12-リポソームの止血能が顕著に向上できることを明らかにした．更に，H12-(ADP）リポソームの安全性に関する結果も併せて報告する．

# The 6th Current Issues on Blood Substitute Research

## October 24－25
## 2008

## Program & Abstracts

**President**
**Koichi Kobayashi（Keio University）**

**Congress Venue**
**Keio University Shinanomachi Campus**
**35 Shinanomachi, Shinjuku-ku,**
**Tokyo Japan**

# *Welcome to the 6th CIBSR*

**Koichi Kobayashi**

*Professor Emeritus, Keio University, School of Medicine*

It is our great pleasure to offer an honorable opportunity to serve as a president of the 6th Current Issues on Blood substitute Research. This meeting is held in conjunction with the 15th Annual Meeting of the Society of Blood Substitutes Japan for which Dr. H. Horinouchi, Associate Professor of School of medicine, Keio University, is the president to give dense scientific sessions to propel the research and development of this field.

Since artificial oxygen carriers should be injected into a blood stream, they cannot be free from adverse effects or unexpected events. The history of artificial oxygen carrier is the history of struggle against unsatisfactory reactions. Gold standard of oxygen therapeutics is allogeneic blood transfusion. Decades ago, it seemed that the benefit of transfusion has been considered superior to the artificial oxygen carrier, although blood transfusion will have a considerable complications. To minimize the undesirable effect of artificial oxygen carrier, either cell-free or cellular, a lot of innovation has been made. It has been always a big question to what extent we can permit the undesirable effect when artificial oxygen carrier is brought in clinical use.

Meanwhile, understanding of vascular reaction to several gases has been deepened since gas mediators were discovered in 90'. These gases seemed transferred to tissue by either specific gas carriers or plasma. Oxygen compounds such as $O_2$, NO, CO are carried by heme-based gas carriers: namely hemoglobin, myoglobin, and cytoplasmic hemeprotein. Research in the field of gas biology will give us new insights and ways to develop artificial oxygen carriers.

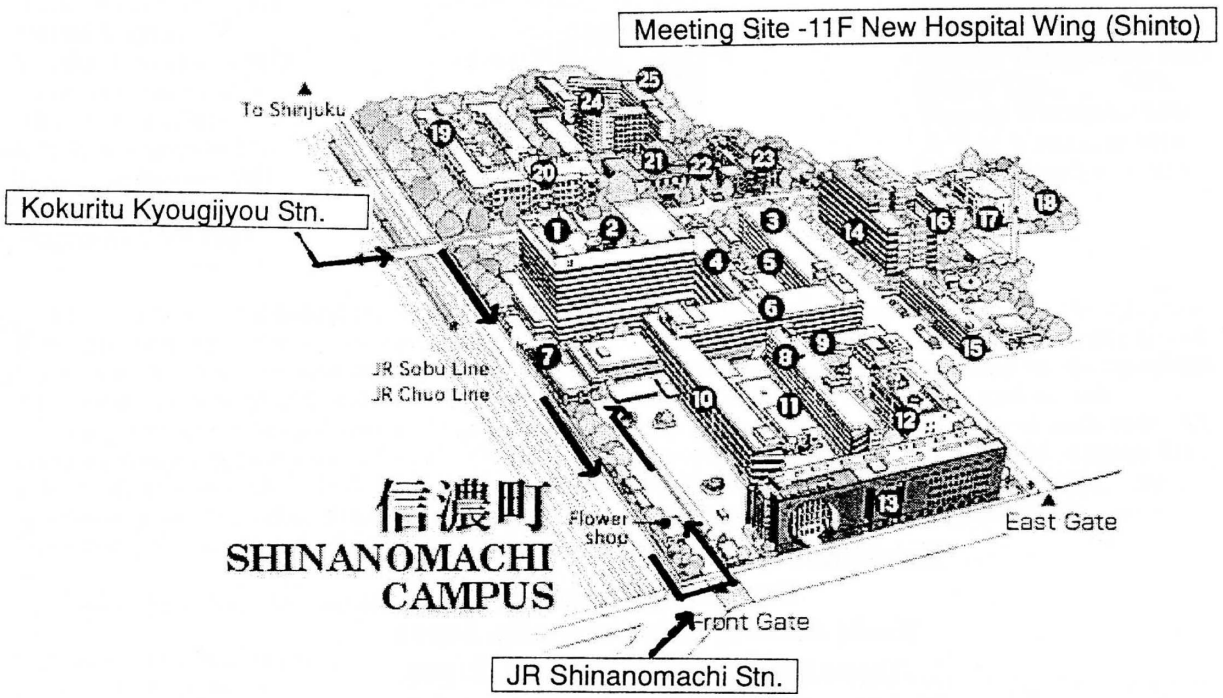
We hope each of you enjoy the meeting and also take the opportunity to enjoy beautiful autumn days in Japan.

# 6th CIBSR Schedule

| October 24 (FRI) New Hospital Wing (Shinto) 11F | | October 25 (SAT) New Hospital Wing (Shinto) 11F | |
|---|---|---|---|
| 8:50 | **Opening Remarks** | 8:30 | **Plenary Lecture II** Warren M. Zapol (Massachusetts General Hospital) |
| 9:00 | **Plenary Lecture I** Makoto Suematsu (Keio University) | 9:10 | **Keynote Lecture II** A. Gerson Greenburg (Biopure Corporation) |
| 9:40 | **Keynote Lecture I** Marcos Intaglietta (Univ. of California, San Diego) | 9:50 | **Coffee Break** |
| 10:20 | **Coffee Break** | 10:00 | **Symposium II** *Acceptable Vascular Reaction in HBOC* |
| 10:30 | **Educational Lecture** Hirosuke Kobayashi (Kitasato University) | 10:30 | |
| 11:10 | **New Material** | 11:00 | |
| 11:50 | | 11:20 | **Invited Lecture** Jonathan S. Jahr (Univ. of California, Los Angeles) |
| 12:10 | **Lunch Time Symposium** | 12:00 | |
| 13:10 | **Presidential Address** (6th CIBSR) Koichi Kobayashi (Keio University) | 12:30 | **Lunch** |
| 13:40 | | 13:00 | **Oral Session** *Gas Carrier and Gas Biology* |
| 14:00 | **Symposium I** PART I | 13:30 | |
| 14:30 | *Cutting Edge of Research of Artificial Oxygen Carriers* | 14:00 | |
| 15:00 | **Coffee Break** | 14:30 | |
| 15:10 | | 15:00 | **Closing Remarks** |
| 15:30 | **Symposium I** PART II | 15:15 | |
| 16:00 | *Cutting Edge of Research of Artificial Oxygen Carriers* | 15:30 | |
| 16:30 | | 16:00 | |
| 17:10 | | 16:30 | |
| 17:30 | | 17:00 | |
| 18:00 | | 17:30 | |
| 18:30 | **Banquet** Tokyo Dome Hotel | 18:00 | |
| 19:00 | | 18:30 | |
| 19:30 | | 19:00 | |
| 20:00 | | 19:30 | |
| | | 20:00 | |

## Map (Shinanomachi Campus of Keio University)

35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582 Phone +81-(0)3-3353-1211




1. New Hospital Wing
2. University Co-op
3. Wing 7 Wards
4. Wing 6 Wards
5. Radiographic Diagnosis Center
6. Central Wing: 5th Floor= Office of Planning, 2F= Secretarial Office, B1F= Personnel Division, Accounting and Finance Office, Academic Affairs Office, Administrative Affairs Office, Supplies Division
7. Restaurant
8. Clinical Research Institute
9. Clinical Research Hall
10. Wing 1
11. Department of Rehabilitation Medicine and Division ofHospital Information System
12. Junior College of Nursing
13. Rengakan
14. Institute of Integrated Medical Research
15. East Lecture Hall
16. Second Lecture Hall
17. Education and Research Building: 1F= Student Affairs Office
18. Ground
19. Clinical Research Institute
20. Health Consulting Center
21. Media Center (Kitasato Memorial Library)
22. The Waksman Foundation of Japan, Inc.
23. Building for Preventive Medicine and Public Health
24. Nurse Dormitory (Kobai-ryo)
25. Nurse Dormitory (Hakubai-ryo)

## 10月24日（金） October 24（FRI）

**8：50〜9：00 Opening Remarks**

**9：00〜9：40 Plenary Lecture Ⅰ**

Chairperson： Koichi Kobayashi (Keio University)

Biomedical Application of Metabolome Analysis: Systematic Molecular Mining for Gaseous Signal Transducers
Makoto Suematsui (Keio University)

**9：40〜10：20 Keynote Lecture Ⅰ**

Chairperson： Hiromi Sakai (Waseda University)

Microvascular Reactions in Designing Oxygen Carrying Blood Substitutes
Marcos Intaglietta (University of California, San Diego)

**10：20〜10：30 Coffee Break**

**10：30〜11：10 Educational Lecture**

Chairperson： Hirohisa Horinouchi (Keio University)

Physiological Aspects of Oxygen Transport to Tissue
Hirosuke Kobayashi (Kitasato University)

**11：10〜11：50 New Material**

Chairperson： Shinji Takeoka (Waseda University)

NM-1 Hemostatic Effects of Liposome Carrying Fibrinogen $\gamma$-Chain Dodecapeptide on the Surface and Adenosine 5'-Diphosphate Inside as a Platelet Substitute
Makoto Handa (Keio University)

NM-2 $O_2$ Binding Properties of Recombinant Albumin-heme Complexes Having Arginine at the Entrance of the Heme Pocket
Akito Nakagawa (Waseda University)

**12：10〜13：10 Lunch Time Symposium**

Chairperson： Koichi Kobayashi (Keio University)

LS-1 Hb-vesicle, a Cellular Hb-based Oxygen Carrier, Fulfills the Physiological Roles of the RBC Structure
Hiromi Sakai (Waseda University)

LS-2 The Effect of Hemoglobin Vesicle Administration on Ventilator Induced Lung Injury
Yotaro Izumi (Keio University)

LS-3 Effect of Artificial Oxygen Carrier Hb Vesicle on Cerebral Blood Flow During and After Hemodiluted Cardiopulmonary Bypass in Rat
Ryo Aeba (Keio University)

**13：10〜13：40 Presidential Address**

Chairperson： Eishun Tsuchida (Waseda University)

Will Our Dream "Clinically Applicable Artificial Oxygen Carrier" Come True in Foreseeable Future?
Koichi Kobayashi (Keio University)

**13：40〜15：00 Symposium I** ***Cutting edge of research of artificial oxygen carriers (PART 1)***

PART I Chairperson： Yotaro Izumi (Keio University)

SY-I-1 Second Generation PEGylated Hbs with a Range of $O_2$ Affinities to Improve Tissue Oxygenation without Activating Autoregulation Induced Vasoconstriction
Seetharama A. Acharya (Albert Einstein College of Medicine of Yeshiva University)

SY-I-2 Effect of the Initially Injected Hemoglobin Vesicles (HbV) on the Pharmacokinetics of Second Injection of HbV in Mice
Kazuaki Taguchi (Kumamoto University)

SY-I-3 Small Volume Resuscitation from Hemorrhagic Shock with Polymerized Bovine Hemoglobin
Pedro Cabrales (La Jolla Bioengineering Institute)

SY-I-4 Liposome-encapsulated Hemoglobin Transfusion Rescues Rats Undergoing Progressive Hemodilution from Lethal Organ Hypoxia without Scavenging Nitric Oxide
Yashiro Nogami (National Defense Medical College)

**15：00〜15：10 Coffee Break**

**15：10〜17：10 Symposium I** ***Cutting Edge of Research of Artificial Oxygen Carriers (PART 2)***

PART II Chairperson： Amy Tsai (University of California, San Diego)

SY-I-5 Pegylation Destabilizes the Haemoglobin Tetramer
Luca Ronda (University of Parma)

SY-I-6 Vanished Oxygen Affinity of Myoglobin by Pyridine-containing Heme
Saburo Neya (Chiba University)

SY-I-7 Hemospan®: Rational Design, Preclinical Effects, and Clinical Development of PEG-conjugated Human Hemoglobin
Mark A. Young (Sangart, Inc.)

SY-I-8 Transient Induction of Immune-suppressor Cells in Rat Spleen by Massive Injection of Hemoglobin-vesicle (HbV)
Hiroshi Azuma (Hokkaido Red Cross Blood Centre)

SY-I-9 Potential Tumor Oxygenation by Systemic Administration of Hemoglobin Vesicle in a Mouse Lewis Lung Carcinoma Model
Yotaro Izumi (Keio University)

SY-I-10 Polymerized Placenta Hemoglobin Improves Cardiac Functional Recovery and Reduces Infarction Size of Isolated Rat Heart
Qian Yang (Chengdu Medical College)

**18：30〜 Banquet at Tokyo Dome Hotel**

## 10月25日（土） October 25（SAT）

**8：30〜9：10 Plenary Lecture II**

Chairperson： Koichi Kobayashi (Keio University)

Prevention of the Pulmonary Vasoconstrictor Effects of HBOC-201 in Awake Lambs by Continuously Breathing Nitric Oxide
Warren M. Zapol (Massachusetts General Hospital)

**9：10〜9：50 Keynote Lecture II**

Chairperson： Masuhiko Takaori (Higashitakarazuka Sato Hospital)

HBOCs and Vasoactivity: An Alternate Perspective
A. Gerson Greenburg (Biopure Corporation and Brown University)

**9：50〜10：00 Coffee Break**

**10：00〜11：20 Symposium II** ***Acceptable Vascular Reaction in HBOC***

Chairperson： Hae Kim (Brown University)

SY-Ⅱ-1 Hemoglobin-Based Oxygen Carrier Mediated Vasoactivity : Proposed Mechanisms and Potential Remedies
Hae W. Kim (Brown University)

SY-Ⅱ-2 Does Lowering Oxygen Affinity of Polyethylene Glycol Conjugated Hemoglobins Cause Arteriolar Vasoconstriction?
Amy G. Tsai (University of Carifornia, San Diego)

SY-Ⅱ-3 Mechanism to Avoid Vasoconstriction and Maintain Perfusion of Pegylated Hemoglobins
Pedro Cabrales (La Jolla Bioengineering Institute)

SY-Ⅱ-4 Effect of Artificial RBCs on Murine Hemorrhagic Shock Model
Yutaka Tomita (Keio University)

**11：20〜12：00 Invited Lecture**

Chairperson：Hiroshi Morisaki (Keio University)

Clinical and Translational Advances in HBOCs: One Investigator's Perspective
Jonathan S. Jahr (University of California, Los Angeles)

**12：00〜13：00 LUNCH**

**13：00〜15：00 Oral Session** ***Gas Carrier and Gas Biology***

Chairperson： Teruyuki Komatsu (Waseda University)

S-1 Design and Evaluation of S-nitrosylated Human Serum Albumin as a Novel Anticancer Drug
Toru Maruyama (Kumamoto University)

S-2 Hemoglobin-vesicles as $O_2$- and CO-carriers
Hiromi Sakai (Waseda University)

S-3 Injectable O-15 System for Oxygen Metabolism Studies Using Hemoblobin-vesicle (HbV): Automatic Labeling and Application in Rats Using PET
Vijay Narayan Tiwari (University of Fukui)

S-4 Comparison of Nitric Oxide-induced Oxidation of Recombinant Oxyhemoglobin Subunits Using a Competition Experiment
Yen-Lin Lin (National Chung Cheng University)

S-5 Polymerized Human Placenta Hemoglobin Decreased the Pathological Nitric Oxide Production in Cold Stored Rat Heart
Tao Li (Sichuan University)

S-6 Polymerized Human Placenta Hemoglobin Ameliorates Oxidative Stress and Energy Deficiency in Cold Stored Rat Heart
Tao Li (Sichuan University)

**15：00～15：15 Closing Remarks**

# Poster

PO-1 Assessment of Inflammation-altered Intestinal Permeability Using Arterially Perfused Murine Jejunal Loop
Premysl Bercik (McMaster University)

PO-2 Hemoglobin Encapsulation in Vesicles Retards the Reaction with NO by Intracellular Diffusion Barrier
Hiromi Sakai (Waseda University)

PO-3 Effect of HbV as a Resuscitation Fluid in Uncontrolled Hemorrhage-shock Model: Blunt Kidney Injury Model
Yasuhisa Seishi (Keio University)

PO-4 Resuscitation of Hemorrhagic Shock due to Uncontrolled Hemorrhage-effect of Hemoglobin-vesicle in Vascular Injury Model
Hirohisa Horinouchi (Keio University)

# NOTE

Presidential Address

# Will Our Dream "Clinically Applicable Artificial Oxygen Carrier" Come True in Foreseeable Future?

**Koichi Kobayashi, President of the Society of Blood Substitutes Japan**

*Dept. General Thoracic Surgery, School of Medicine, Keio University, Japan*

*E-mail: kobayash@sc.itc.keio.ac.jp*

In 20th Century, blood transfusion was one of the major therapeutic achievements, but still we have been facing difficult task to keep transfusion medicine safe and reliable. Looking at the newly emerged blood borne infectious diseases and shortage of donor, developing artificial blood component will promise the progress of medical science in 21st Century. Clinical trials of various kinds of cell-free HBOCs are underway especially in North America. Some were suspended and some are now in the final stage. However, the Natanson's paper recently published in JAMA was very surprising. Meta-analysis of cell-free HBOCs clinical data showed that they increase the risk of myocardial infarction and death. The Food and Drug Administration of the United States held a public workshop entitled "Safety of Hemoglobin-Based Oxygen Carriers (HBOCs)" in the end of April 2008. Recent results of clinical trials, safety issues of clinical and preclinical studies, remedies to the side effects, and future directions were discussed. In response to the Natanson's paper, some argument arose. Whatever, it has to be emphasized that HBOCs are necessary for unmet medical needs; a situation where safe RBC transfusion is not available.

Research and development of Hb-vesicle (HbV) and synthetic hemes have been performed by close collaboration of Waseda and Keio for more than 20 years. HbV is a cellular HBOC and it is expected that toxic effects of cell-free HBOCs would be shielded by the cellular structure. Even though we have not come to a clinical stage yet, the preclinical results of our safety and efficacy evaluations of HbV make us confident in advancing to further development of HbV for eventual realization.

Whether cell-free, or cellular HBOCs, I hope a clinically available oxygen carrier to save life and alleviate human suffering will appear in foreseeable future.

# NOTE

Keynote Lecture I

## Microvascular Reactions in Designing Oxygen Carrying Blood Substitutes

**Marcos Intaglietta, Amy G. Tsai, Pedro Cabrales, Beatriz Y. Salazar Vazquez**

*Department of Bioengineering, University of California San Diego, CA, USA*

The effective restitution of blood volume and oxygen carrying capacity to the circulation requires that the microcirculation, where blood exerts its primary role in gas and material exchange remains functional in the presence of changes of the composition of blood. This requires normal capillary perfusion (functional capillary density, FCD, capillaries with the passage of red blood cells), a parameter that must be above a critical threshold to insure tissue survival. The key factor in maintaining FCD is capillary pressure which is directly and linearly related to FCD. Capillary pressure is the resultant of central blood pressure and how this is transmitted from the major blood vessels through the arteriolar microcirculation, which depends on microvessel vasoactivity and blood viscosity, factors directly affected by the local interaction of blood and the endothelium. Maintenance or increased plasma viscosity is beneficial in resuscitation when the reduction of hematocrit lowers blood viscosity, maintaining and/or increasing shear stress on the endothelium and the production of nitric oxide (NO) and prostacyclin. Addition of oxygen carrying capacity with hemoglobin based oxygen carriers must insure that the natural tendency of hemoglobin to scavenge NO is balanced by its production via other mechanisms. Increased shear stress and nitrate reductase activity provide such a balance. Over-oxygenation of the arteriolar walls due to facilitated diffusion and low affinity oxygen carriers also constitute vasoconstrictive mechanisms that impair microvascular function. Optimal microvascular function is determined by a varying combination of oxygen transport properties and mechanical factors and not solely on blood's intrinsic oxygen carrying capacity. This leads to effective resuscitation via mechanism that emphasize either restoration of oxygen carrying capacity or improvement of microvascular function, a situation that in many instances diminishes the need of restituting intrinsic oxygen carrying capacity, if microvascular functionality can be augmented, as evidenced by retransfusion procedures that promote vasodilatation.

# NOTE

Keynote Lecture Ⅱ

## HBOCs and Vasoactivity: An Alternate Perspective

**A Gerson Greenburg**

*VP Medical Affairs, Biopure Corporation; Professor of Surgery Emeritus, Brown University, RI, USA*

The observation of vasoactivity, often referred to as "hypertension" in the literature—in reality an elevation of blood pressure—has plagued the development of hemoglobin based oxygen carriers, HBOCs, for over 20 years. Regulatory agencies have considered it the underlying basis for the emergence of many of the significant and durable serious adverse events (SAE), e.g. myocardial infarction, stroke, renal failure and death. Licensing and even approval for clinical trials has been withheld with vasoactivity expressed as the reason.

HBOC-201, Hemopure®, was evaluated in regulatory agency specified preclinical protocols of organ flow, organ oxygenation and microcirculation and in specific studies of coronary flow and myocardial function in both animals and humans. The objective of the studies was to determine whether there is vasoactivity and to what extent it can be localized to organs of concern. In exchange models skeletal muscle was the only bed showing vasoconstriction, an observation substantiated by the tissue oxygen and microcirculation studies. Top loading in patients with coronary artery disease did not induce coronary vasoconstriction (direct observation of coronary function) and perfusion of an occluded coronary artery for 3 minutes with oxygenated HBOC-201 preserved function fully without evidence of ischemia. These, and other studies, demonstrate that vasoactivity is not the operative agent in the emergence of SAEs in a pivotal Phase 3 orthopedic surgery trial. By properly done subset analysis of the 350 patients treated in this trial, the safety profile of HBOC-201 was equal to that of blood when 3 or fewer units of red cells were used. In this trial elevations in blood pressure were observed but only a few at levels that required treatment. SAEs were not associated with the blood pressure changes.

In a series of 54 patients treated with HBOC-201 for various "anemia" indications, there were a few instances of elevated blood pressure, investigators made aware of the possibility beforehand, and these were successfully treated with nitrite donors, bets-blockers or calcium channel blocker with good results. In some instances, an elevation in blood pressure may be helpful to insure tissue perfusion. There is evidence in models of traumatic brain injury that the elevation in pressure is beneficial in an otherwise lethal model. Moreover, there is evidence in the treatment of human stroke that survival and improved recovery are achieved with elevated blood pressure. A new definition of "hypotension" has been proposed with systolic below 110 considered detrimental for hypovolemic shock. Have we been generally under-resuscitating patients?

Vasoactivity is a known side effect of this class of agents. Evidence is accumulating to indicate it may not be the basis for the emergence of SAEs. Optional explanations will be presented.

# NOTE

Plenary Lecture I

## Biomedical Application of Metabolome Analysis: Systematic Molecular Mining for Gaseous Signal Transducers

**Makoto Suematsu**

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Gaseous metabolites such as NO, CO and $H_2S$ are too small to handle for mining their receptors through nanobeads technology. We have recently applied metabolome analysis based on CE-MS that allowed us to collect systematic information on small molecular metabolites responding to disease conditions. Using differential metabolomics analyses, we compared alterations in the metabolites, the molecular size of which are less than 1000, between normal and the acetaminophen (AAP)-induced liver injury model using mice where CO was overproduced via heme oxygenase (HO) through cytochrome P450 degradation. Results indicate increases in methionine and S-adenosylmethionine in the liver and hyperhomocystinemia, and decreases cystathionine, cysteine and GSH. Although the decreases in transsulfuration metabolites appeared to result from consumption of thiols for the xenobiotic detoxification, mechanisms for the increases in remethylation metabolites remain to be solved. Since the increases in the remethylation metabolites were inhibited by blocking HO through Zn-protoporphyrin-IX, we hypothesized that CO generated by HO might inhibit the heme-containing cystathionine $\beta$-synthase (CBS), the rate-limiting enzyme for transsulfuration pathway. As expected, the recombinant enzyme was inhibitable by CO but not by NO. These results suggest that the CO-CBS system serves as a protective mechanism against xenobiotic detoxification. Moreover, heterozygous CBS-knockout mice lacked CO responsiveness for regulating transsulfuration pathway. To note is that the inhibitory action of CO on CBS is stabilized by $H_2S$, an end product of the enzyme, suggesting a product inhibition mechanism operated by multiple gases. Metabolome analysis appears a powerful strategy for mining gas-responsive signal transducers in vivo. A variety of biomedical application of this technology for *Gas Biology* will be shown in the lecture.

# NOTE

Plenary Lecture Ⅱ

## Prevention of the Pulmonary Vasoconstrictor Effects of HBOC-201 in Awake Lambs by Continuously Breathing Nitric Oxide

**Binglan Yu, Gian Paolo Volpato, Kenneth D. Bloch, Warren M. Zapol**

*Anesthesia Center for Critical Care Research, Department of Anesthesia and Critical Care, Harvard Medical School at Massachusetts General Hospital, MA, USA*

Background:
Hemoglobin (Hb)-based oxygen-carrying solutions (HBOC) provide emergency alternatives to blood transfusion to carry oxygen to tissues without the risks of disease transmission or transfusion reaction. Two primary concerns hampering the clinical acceptance of acellular HBOC are the occurrence of systemic and pulmonary vasoconstriction and the maintenance of the heme-iron in the reduced state ($Fe^{+2}$). We recently demonstrated that pretreatment with inhaled nitric oxide (NO) prevents the systemic hypertension induced by HBOC-201 (polymerized bovine Hb) infusion in awake mice and sheep without causing methemoglobinemia. In this study, we investigated the pulmonary and systemic hemodynamic effects of breathing NO both before and after the administration of HBOC-201 in awake lambs.

Methods and Results:
Intravenous administration of HBOC-201 (12 ml/kg; Biopure Corporation, Cambridge, MA) in healthy, awake lambs induced prolonged systemic and pulmonary vasoconstriction. Pretreatment with inhaled NO (80 parts per million (ppm) for 1 h) prevented the HBOC-201-induced increase in mean arterial pressure, but not the increase of pulmonary arterial pressure, systemic vascular resistance, or pulmonary vascular resistance. Pretreatment with inhaled NO (80 ppm, 1 h) followed by breathing a lower concentration of NO (5 ppm) during and after HBOC-201 infusion prevented systemic and pulmonary vasoconstriction without increasing methemoglobin levels.

Conclusions:
These findings demonstrate that pretreatment with inhaled NO followed by breathing a low concentration of NO during and after administration of HBOC-201 may enable administration of an acellular Hb substitute without vasoconstriction while preserving its oxygen-carrying capacity.

# NOTE

Invited Lecture

# Clinical and Translational Advances in HBOCs: One Investigator's Perspective

**Jonathan S. Jahr**

*Professor of Clinical Anesthesiology*
*David Geffen School of Medicine at UCLA, CA, USA*

Extensive work from my laboratories over the past twelve years in translational and clinical models has resulted in significant publications [1 - 5, 7 - 19]. Clinical Phase I, II, and III trials with Biopure's Hemopure, Hemosol's Hemolink, and Alliance's Perflubron have produced useful data, although both Hemosol and Alliance have not pursued these products, probably due to poor study designs in cardiac surgery models, and lack of cooperation with independent investigators (refusal to publish existing data, etc) (1,2). This has been reviewed (3,4). Northfield's PolyHeme and Sangart's Hemospan are moving forward with Phase II and III trials, but have been reluctant to let independent investigators test product critically (5,6). A recent meta-analysis has compared mortality and morbidityof the current products (Hemopure, PolyHeme, and Hemospan and older discontinued products, Hemolink and HemeAssist) not to primary or secondary endpoints, but to mortality, all derived from post-hoc subset data analysis (6, 6a)

In our translational laboratories, large and small animal experiments have been important to delineate product efficacy of oxygen delivery and safety in four HBOCs from three manufacturers independently (7 - 19).

In a canine hypovolemia resuscitation model, Oxyglobin reconstituted splanchnic perfusion and oxidatative metabolism in spite of pronounced systemic vasoconstriction and insufficient restoration of cardiac output and $DO_2$; it may improve diffusive oxygen transport in the microvasculature by virtue of hemodilution an its high efficiency in the uptake and release of oxygen (7)

In the same model as reference 7, a study confirmed that real-time (in vivo) microvascular studies, which were conducted only in small rodent models in the past, can be performed simultaneously with systemic function, hemodynamic, and oxygenation studies in a large animal model for relevant data correlation (8).

In a similar canine model that included different resuscitation strategies after hemorrhagic shock, Oxyglobin infusion may not improve oxygen delivery more than hetastarch, likely due to hemodilution caused by its high colloid oncotic pressure, but may facilitate diffusive oxygen transport to tissues (9)

In a more advanced canine model of hemorrhagic shock with pre-hospital and hospital resuscitation modes, including use of vasopressin, we concluded that low-volume crystalloid or Oxyglobin resuscitation posthemorrhage may improve (but not restore) macro- and microvascular functions and tissue oxygenation, while arginine vasopressin infusion may only improve blood pressures and result in lower overall systemic perfusion compared with low-volume saline or hemoglobin glutamer-200 treatment and worsening of anaerobic conditions in skeletal muscle (10).

In an attempt to use HBOC's to estimate circulating blood volumes, we concluded that circulating plasma volume and circulating blood volume may be accurately estimated by using plasma hemoglobin concentration measurements after HBOC infusion (11).

In a rabbit model using magnetic resonance imagining, noninvasive in vivo magnetic resonance measurement of oxygen saturation is valid for whole blood mixed with stroma-free hemoglobin. Therefore, magnetic resonance oximetry may be clinically useful for assessing oxygenation status in patients resuscitated with an HBOC (12).

OxyVita, a new generation HBOC, and Oxyglobin, HBOCs with different molecular weights, had similar effects on coagulation as measured by thrombelastography. The impairment of coagulation by HBOCs and hetastarch occurred at doses corresponding to 12 mL/kg or a blood volume replacement of 17%. The use of HBOCs at doses corresponding to 23 mL/kg or a blood volume replacement of 33% significantly decreased coagulation to levels associated with increased clinical bleeding in this preliminary study. Minimal coagulopathic effects are expected with use of OxyVita at the manufacturer's anticipated effective dose of 10 g or 2 to 3 mL/kg (13).

In a clinical laboratory, effects of Hemopure on coagulation testing were evaluated; mechanical detection methods (fibrometer, STA, CS-190) and the MDA-180 methods were less affected by increasing levels of Hemopure than optical detection devices for all test parameters (14)

In measuring hemoglobin, using a point of care device the HemoCue Plasma/Low Hemoglobin System is a reliable instrument for detecting and measuring small concentrations of three different HBOCs in plasma (15). The B-hemoglobin photometer accurately determined the concentration of three HBOCs solutions dissolved in canine plasma.

The validity of arterial and mixed venous oxygen saturation measurements in a canine hemorrhage model after resuscitation with varying concentrations of hemoglobin-based oxygen carrier was determined (16). We concluded that the administration of this oxygen therapeutic may interfere with the values of some monitors.

Lactate measurements with three HBOCs were evaluated, and based on the samples tested in this study, results indicate true lactate levels in the presence of HBOC-200 may be consistently underestimated when measured in a spectrophotometry-based lactate analyzer, especially at higher lactate concentrations. The clinical implications of this study are that with increasing levels of an HBOC in human plasma, lactate interpretation may become inaccurate when measured by a spectrophotometric lactate analyzer, causing underestimation of "true" lactate values and possible under-treatment of the patient. Therefore, caution must be exercised when interpreting lactate results from the Synchron LX® 20 when a HBOC is present in plasma (17).

Methemoglobin concentration increases in patients receiving HBOCs. When interpreting lactate concentrations from a patient with an HBOCpresent in plasma, underestimation of true lactate levels may occur unrelated to methemoglobin concentrations (18).

High methemoglobin concentrations in Oxyglobin cause additive coagulation impairment that likely results from the effects of oxidative substances on platelet function and coagulation proteins. Oxidative products adversely react with coagulation factors and modify redoxsensitive sites in the platelets. Therefore, if methemoglobinemia occurs as a result of HBOC administration and if the levels are significantly elevated (greater than 10%), impairment of coagulation is possible (19) .

In summary, work on HBOCs is presented, providing independent validation of safety and efficacy, crucial for FDA and other regulatory registration for any product, and increased acceptance. Independent investigation, regardless of outcome, is crucial for good science to prevail.

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# NOTE

Educational Lecture

# Physiological Aspects of Oxygen Transport to Tissue

**Hirosuke Kobayashi**

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The oxygen metabolism at mitochondria is similar, but there is a considerable diversity in the oxygen transport system among living things. Gill was developed in fish and has a fine counter-current gas exchange system. Lungs were developed from esophagus in amphibians, reptiles, birds and mammals. Bird's lung has a cross-current efficient gas exchange system, while mammals have pool lungs with large gas exchange area. Current topic is that dinosaurs had bird-type lungs and some of them evolved into birds. The oxygen transport in the mammalian lungs is determined by two limiting factors: perfusion limitation and diffusion limitation. In healthy humans, perfusion limitation is the main limiting factor, except at high altitude on exercise, while the diffusion limitation is the main limiting factor in patients with low diffusing capacity, particularly during exercise. Circulation system can also be considered as a part of respiratory system, where oxygen bound to oxygen carrier molecules is transported to microvessels. Arterial vessels and venous vessels are running parallel, and gas exchange occurs between arterial and venous vessel. The Bohr effect is considered to contribute to oxygen transport in the lungs and tissues. It is also possible that peripheral oxygen pressure is stabilized through counter-current gas exchange between microvessels in the presence of the Bohr effect.

# NOTE

SY－Ⅰ－1

## Second Generation PEGylated Hbs with a Range of $O_2$ Affinities to Improve Tissue Oxygenation without Activating Autoregulation Induced Vasoconstriction

**Seetharama A. Acharya[1], Fantao Meng[2], Hongying Liang[2], Marcos Intaglietta[3]**

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HexaPEGylated Hb, $(SP\text{-}PEG5K)_6$-Hb, and its prototype MP4 are vasoinactive. The taming of the vasoactivity of Hb on PEGylation has been attributed to (i) the plasma expander like properties of hexaPEGylated Hb and (ii) high oxygen affinity. The high oxygen affinity minimizes the oversupply of oxygen to vascular walls on the arterial side, thereby limiting the autoregulation mediated vasoconstriction. This high oxygen affinity of hexaPEGylated Hbs, however, makes them function predominantly as plasma expanders. To facilitate better tissue oxygenation, it is necessary to delineate the correlation between the oxygen affinity of PEGylated Hb and tissue oxygenation without activating the vasoconstrictive activity. We have now designed PEGylated Hbs with a range of $O_2$ affinities using low $O_2$ affinity $\alpha\alpha$-fumaryl Hb ($P_{50}$ ~30 mm Hg) as the primary substrate for PEGylation and acylation chemistry mediated PEGylation to engineer optimal plasma expander like properties. The oxygen affinity of $\alpha\alpha$-fumaryl Hb has been modulated either by NEM modification of Cys-93($\beta$) ($P_{50}$ ~12 mmHg) or by carboxymethylation of the $\alpha$-amino group of Val-1($\beta$) ($P_{50}$ ~63 mm Hg). HexaPEGylation of $\alpha\alpha$-fumaryl Hb with site specific carboxymethylation on Val-1($\beta$), $\alpha\alpha$-fumaryl Hb and NEM modified $\alpha\alpha$-fumaryl Hb using SPA-PEG5K generates products with a $P_{50}$ around 42, 20 and 10 mmHg, respectively. All the three hexaPEGylated $\alpha\alpha$-Hb display a molecular radius of about 5.8 nm, and a COP and viscosity at 4% around 53 mmHg and 1.9 cP, respectively. DecaPEGylation of $\alpha\alpha$-fumaryl Hb with site specific carboxymethylation on Val-1($\beta$) with SPA-PEG3K generates a product with a $P_{50}$ ~ 30 mmHg. The oxygen affinity of these four PEGylated products is expected to facilitate the identification of the optimum oxygen affinity and the concentration of Hb needed in the plasma to optimize the tissues oxygenation with minimal impact on the autoregulation mediated vasoactivity.

# NOTE

SY－I－2

## Effect of the Initially Injected Hemoglobin Vesicles (HbV) on the Pharmacokinetics of Second Injection of HbV in Mice

**Kazuaki Taguchi[1], Yukino Urata[1], Makoto Anraku[1], Toru Maruyama[1], Toshiya Kai[1], Daisuke Kadowaki[1], Hiromi Sakai[2], Koichi Kobayashi[3], Eishun Tsuchida[2], Masaki Otagiri[1]**

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Objective: Hemoglobin-vesicle (HbV) is an artificial oxygen carrier and has liposome structure with polyethylene glycol (PEG). Recently, it reported that PEG-liposome lose their long-circulating characteristic when they are administrated twice in the same animals (referred to as "accelerated blood clearance (ABC) phenomenon"), and anti-PEG IgM, which elicits a response by the spleen, plays an essential role in induction of the ABC phenomenon. Therefore, we investigated whether the first dose of HbV at a dose of 0.1mg/kg or 1400mg/kg have an effect on the pharmacokinetic behavior of the second dose in mice. Additionally, we also studied the induction of IgG or IgM against HbV. Method: Seven days after the first injection of non-labeled HbV (0.1 or 1400 mg Hb/kg body weight), the male ddY mice received the 125I-HbV, which labeled the internal Hb of HbV with iodine (the volume was identical to the volume of first injection). The IgG and IgM against HbV were detected by ELISA. Result and Discussion: The second dose was cleared rapidly from the circulation and was dramatically accumulated in liver, as compared to first injection, when mice were received first dose at a dose of 0.1mg/kg, but not 1400mg/kg. However, the amounts of IgM against HbV, which was promoted ABC phenomenon, were produced by administration at a dose of both 0.1mg/kg and 1400mg/kg, while that of IgG against HbV was not detected. These results show that IgM against HbV is elicited by even administration of HbV, and the suppression of ABC phenomenon at a dose of 1400mg/kg would be saturation of phagocytic processing. Therefore, it would need to consider the induction of ABC phenomenon in repeated administration of HbV.

# NOTE

SY−I−3

## Small Volume Resuscitation from Hemorrhagic Shock with Polymerized Bovine Hemoglobin

**Pedro Cabrales[1], Amy Tsai[2], Marcos Intaglietta[2]**

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Systemic and microvascular hemodynamic responses to hemorrhagic shock volume resuscitation with hypertonic saline (HTS) followed by infusion of polymerized bovine Hb (PBH) at different concentrations were studied in the hamster window chamber model, to determine role of plasma oxygen carrying capacity and vasoactivity during resuscitation. Moderate hemorrhagic shock was induced by arterial controlled bleeding of 50% of blood volume (BV), and a hypovolemic state was maintained for one hour. Volume was restituted by infusion of HTS (7.5% NaCl) 3.5% of BV of followed by 10% of BV of PBH at two different concentrations. Resuscitation was followed for 90 minutes and was carried out using 13gPBH/dl [PBH13], PBH diluted to 4gPBH/dl in albumin solution at matching colloidal osmotic pressure (COP) [PBH4], and an albumin only solution at matching COP [PBH0]. Systemic parameters, microvascular hemodynamics and functional capillary density (FCD) were determined during hemorrhage, hypovolemic shock and resuscitation. PBH13 caused higher arterial pressure without reverting vasoconstriction and hypoperfusion. PBH4 and PBH0 had lower MAP and partially reverted vasoconstriction. Only treatment with PBH4 restored perfusion and FCD when compared to PBH13 and PBH0. Blood gas parameters and acid-base balance recovered proportionally to microvascular perfusion. Tissue pO2 was significantly improved in the PBH4 group showing that limited restoration of oxygen carrying capacity is beneficial and compensates for the effects of vasoactivity characteristic of molecular hemoglobin solutions proposed as blood substitutes. Supported by NIH BRP R24 - HL64395, PPG HL71064, grants R01-HL62354, R01-HL76182 and A RMY PR023075.

# NOTE

SY－I－4

## Liposome-encapsulated Hemoglobin Transfusion Rescues Rats Undergoing Progressive Hemodilution from Lethal Organ Hypoxia without Scavenging Nitric Oxide

**Yashiro Nogami[1], Manabu Kinoshita[2], Bonpei Takase[3], Satoshi Syono[4], Shinichi Kaneda[5], Daizou Saitoh[6], Makoto Kikuchi[7], Masayuki Ishihara[8], Tadaaki Maehara[1]**

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**Objective:** To investigate the efficacy of liposome-encapsulated hemoglobin (LHb) transfusion in rats undergoing lethal progressive hemodilution. **Summary Background Data:** Unlike other acellular hemoglobin-based oxygen carriers, LHb has lipid bilayer membranes, similar to mammalian red blood cells (RBCs), which prevent hemoglobin from direct contact with blood components and the endothelium. Acellular hemoglobin has high affinity to nitric oxide (NO), and reportedly, acellular hemoglobin-based oxygen carriers have pressor effects on peripheral vessels, because they behave as NO scavengers. The vasoconstrictor effects of acellular hemoglobin may cause decreased peripheral perfusion in massive hemorrhage, thereby diminishing oxygen delivery. **Methods:** Rats were subjected to blood withdrawal (0.2 mL/min) and simultaneously resuscitated by isovolemic fluid transfusion with either LHb, 5% albumin, or washed rat RBCs for 150 min (n=15 in each group). **Results:** All rats transfused with LHb or RBCs were rescued from lethal progressive hemodilution, whereas none of albumin-transfused rats survived. LHb did not affect the plasma NO metabolite levels, suggesting it was not a potent NO scavenger. LHb also improved hemodilution-induced metabolic acidosis, and reduced exaggerated neuroendocrine responses and injuries to the heart, liver, and kidney. It suppressed expression of hypoxia-inducible factor-1alpha in the liver and kidney, suggesting improvement of hypoxia at molecular response levels. However, neither transfused LHb nor RBCs improved the acute lung injury seen after progressive hemodilution. **Conclusion:** LHb transfusion is very effective in rescuing rats undergoing progressive hemodilution from lethal organ hypoxia without scavenging NO. (*Ann Surg* 2008;248: 310-319)

# NOTE

SY−I−5

## Pegylation Destabilizes the Haemoglobin Tetramer

**Luca Ronda[1], Dario Caccia[2], Raffaella Frassi[2], Michele Perrella[2], Elena Del Favero[3], Stefano Bruno[1], Barbara Pioselli[1], Andrea Mozzarelli[1]**

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Haemoglobin conjugated with 6-7 molecules of 5 kDa polyethylene glycol (PEG) exhibits a prolonged life-time in the plasma circulation. This effect is due to the increase in the molecular weight and hydrodynamic volume, which reduces renal filtration and endothelium permeation. However, haemoglobin PEGylated under aerobic conditions shows high oxygen affinity, low modulation of allosteric effectors and almost no cooperativity. To avoid these changes with respect to HbA the PEG conjugation was carried out under anaerobic conditions. The product exhibits an oxygen affinity, response to allosteric modulators, and cooperativity that are closer to HbA than PEGylated oxy-haemoglobin. The alteration of functional properties is partly explained by an increased dissociation of the PEGylated haemoglobin molecules into dimers as suggested by the concentration dependence of the oxygen affinity and the elution profiles in size exclusion chromatography. The chromatographic pattern is consistent with an altered tetramer-dimer equilibrium due to a decreased rate of re-association of the dissociated subunits into tetrameric species. We demonstrated by SDS-PAGE and MALDI-TOF analyses that PEG conjugation yields a reproducible product with an average of 6.7 ± 1 PEG molecules per Hb tetramer and a predicted broad PEG/haemoglobin ratio. This heterogeneity, possibly common to other PEGylated haemoglobins, needs to be considered to evaluate in vivo physico-chemical and physiological parameters in the perspective of their use as safe blood substitutes.

# NOTE

SY−I−6

## Vanished Oxygen Affinity of Myoglobin by Pyridine-containing Heme

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*[2]Waseda University, Japan*

The iron complex of oxypyriporphyrin, a porphyrinoid containing a keto-substituted pyridine, was coupled with apomyoglobin. The reconstituted ferric myoglobin was found to be five-coordinate without iron-bound water molecule. The anionic ligands such as $CN^-$ and $N_3^-$ bound the myoglobin with high affinities while neutral imidazole did not. The IR observation indicated that azide complex was pure high-spin although the corresponding native protein was in the spin-state equilibrium. The reduced myoglobin was five-coordinate, but exhibited no measurable affinity for $O_2$. The affinity for CO was lowered down to 1/2400 as compared with native myoglobin. These anomalies were ascribed to the deformation in the iron coordination core by a relatively large pyridine unit. The ligand binding analyses for the ferric and ferrous myoglobin suggests that the proximal histidine pulls up the iron atom from the deformed core to reduce the interaction between the iron and exogenous ligands. Similarity of the reconstituted myoglobin with guanylate cyclase, a NO-responsive signaling hemoprotein, was pointed out.

# NOTE

SY−I−7

## Hemospan®: Rational Design, Preclinical Effects, and Clinical Development of PEG-conjugated Human Hemoglobin

**Mark A. Young[1], Peter Keipert[1], Kim D. Vandegriff[1], Robert M. Winslow[1,2]**

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The effectiveness of hemoglobin-based oxygen carriers (HBOCs) to deliver oxygen is dependent on the profile of oxygen affinity, hemoglobin concentration and oxygen content, and cardiovascular actions. The ideal molecular and solution properties for HBOCs and the associated actions in animals and humans remain a subject of intense investigation. Hemospan® is polyethylene-glycol (PEG)-conjugated human hemoglobin tetramer with a large molecular radius and high oxygen affinity (P50 =5 mmHg), formulated at low hemoglobin concentration (4.3 g/dL) and high colloid osmotic pressure (70 mmHg). We have demonstrated that these characteristics limit facilitated diffusion of oxygen from cell free hemoglobin and, as a result, avoid autoregulatory vasoconstriction associated with oversupply of oxygen to the arteriolar smooth muscle. In a number of experimental animal models, inspiration of 100% oxygen induces hypertension and a decrease in cardiac output, effects which are mimicked by $\alpha\alpha$ crosslinked Hb or unmodified stroma-free Hb (SFH). The consequence of vasoconstriction with $\alpha\alpha$ Hb or SFH is to compromise oxygen delivery, acid base status and hemodynamic stability after hemodilution or hemorrhage. In contrast, administration of Hemospan is not associated with vasoconstriction and, instead, maintains oxygen delivery, cardiac output, and acid-base status following hemodilution or severe hemorrhage. Thus Hemospan preserves perfusion and oxygen delivery compared with other HBOCs exhibiting peripheral vasoconstriction. Measurement of perivascular nitric oxide after administration of HBOCs suggests that the lack of vasoconstriction with Hemospan is not due to differential NO scavenging. In addition to the critical lack of vasoconstriction, plasma volume expansion with Hemospan is also important in maintenance of peripheral perfusion and oxygen delivery. Hemospan expands the intravascular volume by approximately 1.6-fold the infused volume, similar to that of 10% pentastarch, but significantly greater than that of $\alpha\alpha$ Hb or the new-generation hetastarch, Voluven. Our studies have demonstrated that the combined effects of oxygen carrying capacity and volume expansion improve hemodynamic stability, acid-base status and survival after hemodilution and hemorrhage relative to other HBOCs or matched colloids.

The clinical development program for Hemospan has strived to capitalize on the hemodynamic stability and oxygen delivery observed in preclinical models. Phase II studies in orthopedic surgery patients demonstrated significantly improved hemodynamic stability and maintenance of arterial pressure during spinal anesthesia. Two Phase III studies were deliberately designed to establish a safety database in the controlled clinical setting of orthopedic surgery, with the avoidance of surgical hypotension as the primary objective. Secondary endpoints were designed to capitalize on the demonstrated hemodynamic stability to determine benefit in composite indices for organ morbidity and ischemia. These trials have recently completed enrollment of approximately 850 patients and data analysis is currently underway.

# NOTE

SY－I－8

## Transient Induction of Immune-suppressor Cells in Rat Spleen by Massive Injection of Hemoglobin-vesicle(HbV)

**Hiroshi Azuma[1], Hideki Abe[1], Daisuke Takahashi[1], Mitsuhiro Fujihara[1], Hiromi Sakai[2], Keitarou Sou[2], Hirohisa Horinouchi[3], Koichi Kobayashi[3], Eishun Tsuchida[2], Hisami Ikeda[1]**

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[Objective] HbV, a liposomal oxygen carrier containing human hemoglobin is one of the candidates for artificial red blood cells. The safety and efficacy of HbV has been evaluated in various aspects. However, its effects on immune system have not been fully estimated. [Materials and Methods] HbV or empty vesicle(EV) was intravenously infused into rats with or without pre-immunization by Keyhole Limpet Hemocyanin (KLH). Then, splenic T cells (or lymph node T cells) derived from the rats at various interval were cultured with Concanavalin A (Con A) and/or KLH. The cell proliferation was evaluated by $^{3}$H-thymidine incorporation into DNA. Expression of high affinity IL-2 receptor (CD25) on T cell and IL-2 secretion from T cell were also evaluated by flow cytometry. In some experiment, assay was done in the presence of inducible nitric oxide synthase (iNOS) inhibitor. [Results] Con A(0.3$\mu$g/ml)-induced proliferation of splenic T cells derived from rat loaded with HbV and/or EV was significantly suppressed compared with that from saline-loaded rat. The proliferation of HbV-loaded lymph node T cells was also suppressed but the suppression was not comparable to that of splenic T cells. Secondary response to KLH was also suppressed. When splenocytes were taken 7days after HbV injection, no suppression was observed at all. HbV- or EV- loaded splenocytes suppressed Con A-induced proliferation of saline-loaded splenic T cells. No significant reduction of high affinity IL2 receptor expression and IL2 secretion was observed in HbV-loaded splenic T cells. In addition, iNOS inhibitor was shown to restore T cell proliferation to a certain extent. [Conclusion] Massive liposome injection induced immune-suppressor cells in rat spleen, which lead to transient suppression of splenic T cell proliferative response to Con A and KLH. Nitric oxide appeared to be involved in the suppression.

# NOTE

SY−I−9

## Potential Tumor Oxygenation by Systemic Administration of Hemoglobin Vesicle in a Mouse Lewis Lung Carcinoma Model

**Yotaro Izumi[1], Manabu Yamamoto[1], Ken Takeuchi[1], Masazumi Watanabe[1], Hirohisa Horinouchi[1], Yuji Teramura[2], Hiromi Sakai[2], Shinji Takeoka[2], Eishun Tsuchida[2], Koichi Kobayashi[1]**

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Background: Hemoglobin Vesicle (HbV) is currently being developed as an artificial oxygen carrier. It contains concentrated hemoglobin solution within a phospholipid vesicle. Due to its unique oxygen transporting capabilities in comparison to red blood cells, we hypothesized that it may augment tumor tissue oxygen status. Methods: Lewis lung carcinoma fragments were implanted in the right hindlegs of C57 mice. Once the tumors reached approximately 8 mm in diameter, the animals were randomly assigned to receive systemic infusion of 0.9ml/kg of HbV (HbV group) or saline (saline group). Tumor tissue oxygen tension was measured using phosphorescence decay by Oxyspot before infusion, and up to 40 minutes after infusion. Also, in a separate group of animals, the tumors were irradiated (20 Gy) after the completion of infusion. Results: In the HbV group, tumor tissue oxygen tension increased significantly at approximately 10 to 20 minutes following infusion, after which it retuned to preinfusion range. This increase was not observed in the saline group. Additionally, tumor tissue HIF1alpha level was decreased after HbV administration. There was also a significant tumor growth delay after irradiation in the HbV group compared to the saline group. Discussion: The present study suggests that systemic administration of HbV transiently increases tumor tissue oxygen tension. The effect of irradiation given during this period may be augmented, at least in part, by this effect.

# NOTE

SY−I−10

## Polymerized Placenta Hemoglobin Improves Cardiac Functional Recovery and Reduces Infarction Size of Isolated Rat Heart

**Qian Yang[1], Tao Li[2], Jin Liu[2], Wei Wu[2], Mingliang Yi[2], Chengmin Yang[3]**

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**Objective:** Polymerized human placenta hemoglobin (PolyPHb) allowing transporting more $O_2$ to the hypoxia tissues owing to its higher $O_2$ affinity than adult peripheral blood hemoglobin, as well as having a lower viscosity and smaller mean diameter than human red blood cells, suggest PolyPHb may be helpful to microcirculation perfusion and thereby alleviate myocardial ischemia/reperfusion injury. This study was therefore designed to investigate its protective effect to isolated rat heart. **Methods and Results:** Isolated rat hearts were perfused with Langendorff model, after 30 minutes of baseline, the hearts were arrested and stored by St.Thomas' solution (STS) without (STS group) or with 0.5 gHb/dL PolyPHb (PolyPHb group) at 4 ˚C for 8 hours, then reperfused for 2 hours. Compared with STS group, PolyPHb in STS greatly improved the recovery of left ventricular developed pressure (LVDP, $P<0.01$), maximum LVDP increase and decrease rate ( ± dp/dt, $P<0.01$), coronary flow rate (CF, $P<0.01$). Also, both the cardiac enzyme release, including creatine kinase (CK) and lactate dehydrogenase (LDH), and myocardial infarction size were significantly reduced in PolyPHb group. **Conclusion:** our study provided direct evidence that PolyPHb was beneficial to improving cardiac functional recovery and reducing myocardial infarction of 8-hour hypothermic stored rat heart.

# NOTE

## SY−Ⅱ−1 Hemoglobin-based Oxygen Carrier Mediated Vasoactivity : Proposed Mechanisms and Potential Remedies

**Hae Won Kim, Chi-Ming Hai**

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A key impediment to regulatory approval of HBOCs appears to be an undesirable BP elevation (hypertension) often observed following IV administration in preclinical and clinical studies. The HBOC-mediated hypertension, as it occurs without increased cardiac output, appears to be primarily mediated by systemic vasoconstriction. In fact, a recent statement by FDA indicates that all HBOC products reviewed are vasoactive at the doses proposed for clinical use. Because vasoconstriction can cause subnormal blood flow to affected organs, a crucial question is whether HBOC administration could lead to critical organ dysfunction. However, there has not been any published report that definitively links HBOC to observed serious organ dysfunction. While potential benefits of moderate hypertension in certain patient groups (e.g., hemorrhagic shock) is being debated, an acute BP elevation could be detrimental in vulnerable patients. Acceptable vasoactivity is difficult to define generally since it depends on indications and individual patient's condition. Vascular response to HBOCs may also be influenced by such factors as underlying pathologies, concurrent medications, and infusion protocol. The etiology of HBOC-mediated vasoactivity appears to be complex and may involve multiple pathways. Currently proposed mechanisms include HBOC interference with endothelial NO, vascular autoregulatory mechanisms, adrenergic receptors and activation of endothelin and other vasoactive agents. Regardless of mechanisms, the HBOC-mediated hypertension could generally be modulated by many conventional anti-hypertensive drugs including nitrovasodilators, $Ca^{++}$ channel blockers, ACE inhibitors. It may also be possible to prevent the effect by pre- or co-administration of proper vasodilators such as inhaled NO and nitrites. However, metHb formation (non-oxygen carrier) and difficulty of proper dosing in hemodynamically unstable patients would pose significant challenges. This presentation will primarily focus on mechanistic aspect of HBOC-mediated vasoactivity and potential biochemical/pharmacologic means of modulation.

# NOTE

SY－Ⅱ－2

## Does Lowering Oxygen Affinity of Polyethylene Glycol Conjugated Hemoglobins Cause Arteriolar Vasoconstriction?

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Microvascular autoregulatory mechanisms prevent exposure of the tissue to extreme levels of oxygen. The site of this control is the arterioles which vasodilate and vasocontrict thereby allow and restrict oxygen delivery to tissue respectively. Sensitivity of these vessels to hyperoxia led to the design of high oxygen affinity acellular hemoglobins. Their aim was to protect the arterioles from exposure to high oxygen levels and to target oxygen delivery to anoxic tissue regions. Polyethylene glycol conjugated hemoglobins (PEG-Hb) are ideally suitable since their P50 is very low (approximately 4 - 6 mmHg). However in some clinical situations, it maybe advantageous to deliver oxygen to tissue before it becomes nearly anoxic. The objective of these studies was to determine if an increase in oxygen affinity of a PEG-Hb would result in vasoconstriction as well as delivery oxygen to tissue. Microvascular studies were performed with the awake hamster window model. Vasoactivity was studied in a topload experiment using PEGylated alpha-alpha Hb molecule which has a much higher P50 of 30 mmHg compared to first generation PEG-Hbs. Results show that arteriolar vasoconstriction obtained when the microcirculation is exposed to approximately 0.5 g/dl alpha-alpha Hb (P50 = 23 mmHg) is eliminated when the same molecule is PEGylated. Mean arterial pressure was slightly elevated without vasoconstriction. Extreme exchange transfusion experiments with the PEGylated alpha-alpha Hb were also performed to determine if tissue oxygenation is increased by this PEGylated alpha-alpha Hb.

Research supported in part by NIH HL064395, NIH HL071064 and HL062318.

# NOTE

## SY−Ⅱ−3 Mechanism to Avoid Vasoconstriction and Maintain Perfusion of Pegylated Hemoglobins

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Despite the advances in oxygen carrier blood substitutes, the development of materials that effectively maintain blood volume, perfusion and oxygen delivery remain the priority for emergency care and trauma. There is increasing evidence that blood transfusions per se, might not be the optimal blood replacement fluid. Vasoconstriction due to nitric oxide (NO) scavenging and oxygen autoregulation are the problem of small hemodynamic radius hemoglobin (Hb) based oxygen carriers (HBOC). Conversely, Hb based materials with large hydrodynamics radius due to Hb encapsulation and Hb surface conjugations of polyethylene glycol (PEG) are vasoinactive. PEGylated Hb (PEG-Hb) traps water creating a shielding around the Hb, eliminating hypertension and vasoconstriction and even inducing vasodilation. These effects may be related to PEG-Hb's NO management, namely: i) Shear stress related production of NO by mechanotransduction due to increased plasma viscosity and direct molecular interaction; ii) NO transport in an allosterically controlled reversible chemical reaction, and, iii) Nitrite reductase activity of the PEG-Hb. Additionally to increase mechanical interaction with the vascular endothelium, PEG-Hb novel biochemical properties produces intermediates that extract an electron from nitrite, leading to the formation of reaction residues which generate nitrosothiols. The concerns with these mechanisms are: i) How do these reactions generate bioactive NO? ii) How does free NO arrive to the site of action without reacting or being sequestered?; and, iii) If free NO does not form nitrosothiols, how are these being generated? These NO generating mechanisms attributed to PEG-Hb/NO interaction are the pathways for PEGylated Hbs to remain vasoinactive, and may in some conditions, cause them to act as vasodilators, even though PEG-Hb is an effective NO scavenger.
Supported by NIH BRP R24-HL64395, PPG HL71064, grants R01-HL62354, R01-HL76182 and ARMY PR023075.

# NOTE

SY－Ⅱ－4

## Effect of Artificial RBCs on Murine Hemorrhagic Shock Model

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Purpose: Effect of artificial RBCs[1)] injections on brain oxygen tension after withdrawal of blood from the mice was examined. Methods: Under isoflurane anesthesia, a cranial window was opened above the left parieto-temporal cortex of the head of C57BL/6J mice (n=8). An oxygen electrode (Eikohkagaku) was placed in an avascular area near the branch of left MCA of the brain surface. A femoral artery and a tail vein were catheterized. After withdrawal of 0.3-0.5 ml blood, the same amount of saline (n=3) or artificial RBCs (n=5) was systemically injected. During injection, the changes of brain microvasculature, blood pressure (Muromachi) and oxygen tension ($PO_2$) were observed. The movement of FITC-labeled RBCs running through single capillaries in the ROI of the brain parenchyma (50$\mu$m depth) was monitored continuously with video camera (30 frames/s) or high speed laser scanning confocal fluorescent microscope (500 frames/s)[2)]. Results: The control values of blood pressure and $PO_2$ at the brain surface were 80.5 ± 15.5 mmHg and 32.9 ± 4.1 mmHg respectively. After withdrawal of blood, these values decreased to 25.6 ± 5.6 mmHg and 22.9 ± 2.9 mmHg (statistically significant; $P < 0.05$). Stoppage or decrease of velocities of RBCs in the MCA branch was observed. In the group of saline injection, $PO_2$ at the brain surface elevated transiently then decreased a few minutes later. On the other hand, in the group of artificial RBCs injection, $PO_2$ at the brain surface slightly elevated and showed a transient unstable change, then re-elevated to the basal control level and sustained. Velocities of RBCs in the microvessels recovered. Conclusion: It was suggested that artificial RBCs injections during hemorrhagic shock were effective to prevent profound microcirculatory injury of the brain. References:1) Artif Cells, Blood Substitutes, Biotechnol. 35: 81-91, 2007. 2) Microcirculation. 15: 163-174, 2008.

# NOTE

S−1

## Design and Evaluation of S-nitrosylated Human Serum Albumin as a Novel Anticancer Drug

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**Objective:** In recent studies, the cytotoxic activity of NO has been investigated for its potential use in anticancer therapies. Nitrosated human serum albumin (NO-HSA) may act as a reservoir of NO in vivo. However, there are no published reports regarding the effects of NO-HSA on cancer. Therefore, the present study was undertaken to investigate the antitumor activity of NO-HSA. **Method:** NO-HSA was prepared by incubating HSA, which had been sulfhydrylated using iminothiolane, with isopentyl nitrite (6.64 mol NO/mol HSA). Antitumor activity was examined in vitro using murine colon 26 carcinoma (C26) cells and in vivo using C26 tumor-bearing mice. **Result and Discussion:** Exposure to NO-HSA increased the production of reactive oxygen species in C26 cells. Flow cytometric analysis using rhodamine 123 showed that NO-HSA caused mitochondrial depolarization. Activation of caspase-3 and DNA fragmentation were observed in C26 cells after incubation with 100 $\mu$M NO-HSA for 24 h, and NO-HSA inhibited the growth of C26 cells in a concentration-dependent manner. The growth of C26 tumors in mice was significantly inhibited by administration of NO-HSA compared with saline and HSA treatment. Immunohistochemical analysis of tumor tissues demonstrated an increase in terminal deoxynucleotidyl transferase dUTP nickend labeling-positive cells in NO-HSA-treated mice, suggesting that inhibition of tumor growth by NO-HSA was mediated through induction of apoptosis. Biochemical parameters (such as serum creatinine, blood urea nitrogen, aspartate aminotransferase, and alanine aminotransferase) showed no significant differences among the three treatment groups, indicating that NO-HSA did not cause hepatic or renal damage. **Conclusion:** These results suggest that NO-HSA has the potential for chemopreventive and/or chemotherapeutic activity with few side effects.

# NOTE

S−2

## Hemoglobin-vesicles as $O_2$- and CO-carriers

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Hb-vesicles (HbVs, 250 nm) are artificial $O_2$-carriers that encapsulate concentrated Hb solution in phospholipid vesicles. We confirmed that the resuscitative effect of the HbV fluid was comparable with that of RBC transfusion, though both $O_2$-carrying fluids inevitably showed some oxidative damage to the tissues (Crit Care Med 2004; Shock, in press). Recent reports on cytoprotective effects of exogenous CO, by CO-inhalation and injection of CO-releasing molecules, urged us to test infusion of CO-bound HbV (CO-HbV) and RBC (CO-RBC) to improve tissue viability over that of $O_2$-HbV and $O_2$-RBC. Male Wistar rats were anesthetized with 1.5%-sevoflurane inhalation ($FiO_2$=21%) while spontaneous breathing was maintained. Shock was induced by 50% blood withdrawal from femoral artery. Fifteen min later, they received CO-HbV, CO-RBC, $O_2$-HbV, $O_2$-RBC, or empty vesicles (EV) suspended in 5% rHSA. All groups showed prompt recovery of blood pressure and blood gas parameters just after resuscitation, and survived for 6 h of observation period. However, only EV group showed significant hypotension at 3 and 6 h. Plasma enzyme levels were elevated at 6 h, especially in the $O_2$-HbV, $O_2$-RBC, and EV groups. They were significantly lower in the CO-HbV and CO-RBC groups than in the $O_2$-bound fluids groups. Immunohistochemical staining of 3-nitrotyrosine exhibited less oxidative damage in the liver and lung for CO-HbV and CO-RBC groups. Blood HbCO levels (26-39% immediately after infusion) decreased to less than 3% at 6 h while CO was exhaled through the lung. Both HbV and RBC gradually gained the $O_2$ transport function. Collectively, both CO-HbV and CO-RBC reduced oxidative damage to organs in comparison to $O_2$-HbV and $O_2$-RBC. Adverse and poisonous effects of CO gas were not evident in this experimental model.

# NOTE

S−3

## Injectable O-15 System for Oxygen Metabolism Studies Using Hemoblobin-vesicle (HbV) : Automatic Labeling and Application in Rats Using PET.

**Vijay Narayan Tiwari, Hidehiko Okazawa, Masato Kobayashi, Tetsuya Mori, Shingo Kasamatsu, Yasuhisa Fujibayashi**

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Abstract: Artificial red blood cells have emerged as one of the most exciting and promising developments to substitute normal blood at least for a transient period of time. Evidence from experimental and clinical studies suggests that they are stable enough in vivo and hence can be used as oxygen carrier for diagnostic purposes. Intravenously injectable O-15 preparations could be more efficient in terms of utilization and safety than the established O-15 inhalation method for measuring hemodynamic parameters like cerebral blood flow (CBF), oxygen extraction fraction (OEF) and cerebral metabolic rate of oxygen (CMRO2) in brain using positron emission tomography (PET). This study examined the possibility of developing an injectable O-15 system using Hemoglobin vesicles (HbV) a type of artificial red blood cell and later to measure brain oxygen consumption in rats using O-15 labeled HbV. Though primarily HbV were labeled with O-15 gas using direct bubbling, various other strategies were employed to enhance the labeling efficiency of HbV including the use of a vacuum pump to eliminate helium fraction from target gas, bubbling combined with vortexing and addition of L-Cysteine as reductant to protect hemoglobin molecule from oxidation. L-Cysteine was found to play a significant role in increasing the binding of O-15 with HbV. A maximum radioactivity of 204 MBq/ml of HbV was obtained under optimal conditions. CMRO2 values of 6.8 ± 1.4 mL.min-1.100g-1 (n=5) were obtained in rats using a small animal PET scanner after the injection of O-15 labeled HbV. O-15 labeled HbV can be successfully utilized to measure brain oxygen consumption in rats using PET which indicates that HbV is capable of carrying oxygen in vivo and subsequently in delivering it to cerebral tissue.
Keywords: Hemoglobin-vesicle (HbV), Injectable O-15, L-Cysteine, Labeling efficiency, Positron-emission tomography (PET).

# NOTE

S−4

## Comparison of Nitric Oxide-induced Oxidation of Recombinant Oxyhemoglobin Subunits Using a Competition Experiment

**Yen-Lin Lin, Kuang-Tse Huang**

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A low reaction rate with nitric oxide (NO) is one of the important characteristics of hemoglobin (Hb)-based oxygen carriers. The reaction rate between oxyHb and NO is usually measured by stopped-flow spectrophotometry. However, the reported rates vary due to the difficulty of accurately determining the NO concentration and the limit of the instrument dead time. To circumvent these problems, we developed an experiment using oxymyoglobin (oxyMb) to compete with oxyHb for NO that is released from an NO donor. Determination of the rate constants in the competition experiment no longer depends on accurate measurement of time or NO concentration, since this approach instead measures the ratio of rate constants for the reaction of oxyHb and oxyMb with NO. For recombinant mutant Hb $\alpha$(L29F)$\beta$, the rates for $\alpha$(L29F) and $\beta$ are approximately 150- and 1.7-fold smaller than for wild-type Hb. In conclusion, the competition experiment provides an alternative method for determination of relative reaction rates of recombinant Hb subunits with NO.

# NOTE

S–5

## Polymerized Human Placenta Hemoglobin Decreased the Pathological Nitric Oxide Production in Cold Stored Rat Heart

**Tao Li, Pu Zhang, Jin Liu, Ronghua Zhou, Guohua Li**

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Ischemia, inflammation and heart failure result in induced nitric oxide synthase (iNOS) expression in heart, and the subsequent depression of contractile activity and oxygen consumption has been attributed to nitric oxide (NO) inhibition of mitochondrial respiration, which caused damage to myocardium. Considering hemoglobin was the largest reservoir for oxygen and NO in the body, we speculated that polymerized human hemoglobin (PolyPHb), the polymer of hemoglobin, might have potential influence on the NO produced in ischemia/reperfusion (I/R) injury. Isolated Sprague-Dawley rat hearts were divided into 3 groups: sham group, St.Thomas' solution (STS) group and STS+PolyPHb group, and perfused with Langendorff apparatus. After basal perfused for 30 minutes, the last 2 group hearts were arrested and stored with STS and STS plus PolyPHb (0.5g/dL), respectively, at 4 $^{\circ}$C for 8 hours, then reperfused for 2 hours. The sham group hearts were perfused for 2.5 hours without cold storage. PolyPHb added in STS greatly improved the left ventricle contractile performance during reperfusion, including left ventricular developed pressure (LVDP), maximum LVDP increase (+dp/dt) and decrease rate (-dp/dt). In 3 group hearts, no differences in eNOS expression and eNOS phosphorylation were observed, whereas the iNOS expression in STS group was greatly increased. PolyPHb in STS inhibited the iNOS expression and significantly decreased the NO formation in I/R heart, these results were further proved by the less formation of peroxynitrite ($ONOO^-$) in STS+PolyPHb group. In conclusion, PolyPHb in STS is beneficial to the cardiac contractive function recovery and this effect was partially associated with reduced iNOS-derived pathological NO production.

# NOTE

S–6

## Polymerized Human Placenta Hemoglobin Ameliorates Oxidative Stress and Energy Deficiency in Cold Stored Rat Heart

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Growing evidence indicated that augmented superoxide, nitric oxide (NO) and other free radicals from ischemia/reperfusion (I/R) injury caused the oxidative stress and further contributed to energy deficiency. Excessive NO during I/R injury was implicated to an early reversible inhibition of cytochrome-c oxydase in complex IV, then followed by a persistent inhibition of complex I, which decreased the oxygen availability and ATP synthesis in I/R heart. Our previous studies have demonstrated the cardioprotective effect of polymerized human placenta hemoglobin (PolyPHb) in isolated rat heart, this study therefore was designed to investigated the underlying mechanism of this protective effect. Using Langendorff model, isolated Sprague-Dawley rat hearts were divided into 3 groups: sham group, St.Thomas' solution (STS) group and STS+PolyPHb group. After perfused for 30 minutes as baseline, the last 2 group hearts were arrested and stored with STS and STS plus PolyPHb (0.5g/dL), respectively, at 4 °C for 8 hours, then reperfused for 2 hours. The sham group hearts were perfused for 2.5 hours without cold storage. Compared with sham group, STS group exhibited significant increase in NO production, companied by increased oxidative stress in left ventricular tissue, PolyPHb in STS greatly decreased the NO production and ameliorated the oxidative stress, evidenced by elevated superoxide dismutase (SOD) activity and inhibited malondialdehyde (MDA) formation. Consistent with these results, the mitochondria hydrogen peroxide ($H_2O_2$) content and cytochrome-c release in STS+PolyPHb group were greatly reduced, as compared to STS group. Further, the mitochondria ATP synthesis in STS+PolyPHb group was also significantly higher than that in STS group, which conjointly proved the mitochondria respiratory chain was well preserved by PolyPHb after 8-hour cold storage and 2-hour reperfusion. Therefore, our study demonstrated that the cardioprotective effect of PolyPHb might be associated with attenuation of oxidative stress and restoration of energy synthesis in cold stored rat heart.

# NOTE

NM−1

# Hemostatic Effects of Liposomes Carrying Fibrinogen γ-Chain Dodecapeptide on the Surface and Adenosine 5'-Diphosphate Inside as a Platelet Substitute

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We studied platelet substitutes for treatment of bleeding by focusing on a dodecapeptide; HHLGGAKQAGDV (H12), which corresponded to the fibrinogen γ-chain carboxy-terminal sequence (γ400-411), a specific binding module of the ligand for platelet GPIIb/IIIa complex. In this study, we conjugated H12 to the surface of a liposome into which adenosine 5'-diphosphate (ADP) was included to prepare H12-(ADP)liposome, and evaluated their *in vitro* and *in vivo* characteristics related to platelet hemostatic function.

The effect of H12-(ADP)liposome ($\phi$ 250 ± 80 nm) on agonist-induced platelet aggregation was evaluated on an aggregometer (Hema Tracer T-638, Nico Bioscience, Tokyo). Thrombocytopenic animals (rat or rabbit) were made by busulphan infusion, and a template-guided incision (Quikheel™) was made 1 cm from the tip of tail or ear. The tail or ear was immersed in a 50 mL cylinder of saline and the time taken to stop bleeding was measured. Using H12-liposomes having a contrast dye, iopamidol inside, computed tomography (CT) analysis (eXplore Locus™, GE Healthcare UK, Buckinghamshire, England) was performed to detect the accumulation of the iopamidol-incorporated liposomes to the sites of vascular injury in normal rats.

As compared with the H12-liposomes, the H12-(ADP)liposomes significantly enhanced the collagen-induced platelet aggregation, suggesting that release of ADP from the H12-(ADP)liposome was triggered by platelet aggregation. *In vivo* hemostatic effect was studied by measuring the tail bleeding time of thrombocytopenic rats at 5 min after the intravenous administration of the H12-(ADP)liposomes. The bleeding times of the normal ([platelet] = 8.1 ± 0.9x$10^5$ /μL) and thrombocytopenic rats ([platelet] = 2.0 ± 0.3x$10^5$ /μL) were 178 ± 56 and 682 ± 198 s, respectively. The H12(ADP)liposomes at a dose of 10 mg/kg shortened the tail bleeding time to 349 ± 49 s, whereas the H12-liposomes at the same dose were without effects (572 ± 127 s). The similar hemostatic ability of the H12(ADP)liposomes was also seen in thrombocytopenic rabbits. It was thus suggested that the H12-(ADP)liposomes would amplify the in vivo hemostatic ability by releasing ADP. Indeed, with CT analysis, the H12-liposomes were shown to specifically accumulate at the sites of vascular injury. These results indicated that the H12-liposomes would be a promising candidate as an ideal synthetic platelet substitute that is specifically recruited to and exerts its hemostatic ability at sites of vascular injury.

# NOTE

NM−2

# $O_2$ Binding Properties of Recombinant Albumin-heme Complexes Having Arginine at the Entrance of the Heme Pocket

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In our blood stream, iron(III)-protoporphyrin IX (hemin) released from methemoglobin is bound within a D-shaped hydrophobic cavity in subdomain IB of human serum albumin (HSA) with an axial coordination of Tyr-161 and salt bridges between the porphyrin propionates and a triad of basic amino acid residues (Arg-114, His-146 and Lys-190). We found that a pair of site-specific mutations into the subdomain IB of HSA allows the heme to bind $O_2$; (i) introduction of a proximal His at the Leu-185 position and (ii) substitution of Tyr-161 with non-coordinating Leu (rHSA(HK)). The rHSA-heme could be an artificial $O_2$ carrier acting not only as a red blood cell (RBC) substitute but also as an $O_2$-providing therapeutic reagent. However, the rHSA(HK)-heme showed two $O_2$-binding affinities which are probably due to two different orientations of the heme in rHSA. In this paper, we report new rHSA having Arg at entrance of the heme pocket (146 and/or 190 position) and its effect on $O_2$-binding properties. The introduction of Arg yielded a single $O_2$-binding affinity ($P_{50}$: 6-9 Torr, pH 7.0 at 22°C); the value of which was almost the same as that of RBC (8 Torr). Resonance Raman and infrared spectra of this rHSA-heme revealed that the strained coordination disappeared by the introduction of the Arg at 146 and 190 position. We can conclude that introduction of Arg into the entrance of the heme pocket of rHSA-heme is effective at excluding the low $O_2$-binding affinity conformer.

# NOTE

LS−1

## Hb-vesicle, a Cellular Hb-based Oxygen Carrier, Fulfills the Physiological Roles of the RBC Structure

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Biconcave RBCs deform to a parachute-like configuration to flow through a narrower capillary. This profile is believed to be effective to facilitate the gas exchange. On the other hand, physicochemical analyses have revealed that $O_2$-unloading and binding of Hb are significantly retarded by compartmentalization in RBC. Why nature has selected such an inefficient cellular structure for gas transport? Interestingly, some of the answers to this question have been revised by the research of blood substitutes.

Hb-vesicles (HbV) are artificial oxygen carriers encapsulating concentrated Hb solution (35 g/dL) with a phospholipid bilayer membrane. Concentration of the HbV suspension is extremely high ([Hb] = 10 g/dL, volume fraction, ca. 40 vol%) and it has an oxygen carrying capacity that is comparable to that of blood. HbV is much smaller than RBC (250 vs. 8000 nm), but it recreates the functions of RBCs; (i) The $O_2$ unloading of HbV is slower than that of a cell-free Hb solution; (ii) COP is zero. For a massive dosage, HbV has to be co-injected with or suspended in a plasma substitute such as albumin; (iii) The viscosity of HbV is adjustable to that of blood; (iv) HbV is finally captured by RES, and then degraded and excreted promptly; (v) Co-encapsulation of an allosteric effector regulates $O_2$ affinity; (vi) Hemolysis is minimal during circulation and the lipid bilayer membrane prevents a direct contact of Hb and vasculature; (vii) Reaction of NO is retarded by an intracellular diffusion barrier, and HbV does not induce vasoconstriction.

We admit HbV has only a few days of functional half-life after intravenous injection. On the other hand, the obvious advantages are that it is pathogen-free and blood-type-antigen-free; moreover, it can withstand long-term storage for stockpiling. HbV has a variety of potential applications not only as a transfusion alternative but also as an $O_2$ or CO therapeutic fluid that cannot be attained by the present RBC transfusion.

# NOTE

LS−2

## The Effect of Hemoglobin Vesicle Administration on Ventilator Induced Lung Injury

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Hemoglobin Vesicle (HbV) is currently being actively developed as an artificial oxygen carrier. It contains concentrated hemoglobin solution within a phospholipid vesicle. As a resuscitative fluid, it will potentially be administered to patients receiving care in the intensive care unit, which includes mechanical ventilation. Mechanical ventilation differs in a number of ways from physiological breathing. Lung injury may be affected by positive pressure ventilation, known as ventilator induced lung injury (VILI). In the present study, we studied the effects of HbV administration in a rabbit VILI model. HbV did not affect lung function when administered under spontaneous breathing. In this study, we administered HbV when the lung was mechanically ventilated. Rabbits were mechanically ventilated (tidal volume, 30ml/kg), and 30%, or 60% exchange transfusion was done with HbV, saline, or 5% albumin. Hemodynamics, and blood gas parameters were monitored for 4 hours. The lung was then resected for histology. Wet to dry ratio was also measured. In the 30% exchange groups, there was no apparent change in systemic blood pressure in any of the groups. Arterial oxygen tension tended to decrease gradually, but there was no significant difference between groups. Slight lung edema was observed. There was no significant difference in the wet to dry ration between the groups. The moderate decrease in arterial oxygen tension suggested the presence of ventilation induced lung injury, but there was nothing to indicate that it was aggravated by HbV administration. In the 60% exchange groups, HbV administration tended to improve arterial oxygen tension. (Supported by MHLW of Japan)

# NOTE

LS−3

## Effect of Artificial Oxygen Carrier Hb Vesicle on Cerebral Blood Flow During and After Hemodiluted Cardiopulmonary Bypass in Rat.

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Background

Donor blood continues to be priming for clinical cardiopulmonary bypass (CPB) in neonate and infant to avoid unacceptable level of hemodilution. We have previously demonstrated that hemoglobin vesicle (HbV) has contributed preservation of neurocognitive function after hemodiluted CPB in rat. The mechanism of the effect is to be clarified.

Method

Nine rats subjected CPB (90minutes and 200ml/kg/min of bypass flow), without the use of donor blood priming. Hb V was added in the priming of CPB (Hb V group, n=4), whereas the other 5 animals served as control group. Brain tissue oxygen tension and regional cerebral blood flow (rCBF) were monitored during and after CPB.

Result

In both the groups hematocrit decreased to 13.3+/- 0.5, 12.0+/-1.4 during CPB, but maintained brain tissue oxygen tension during and after CPB. rCBF increased in both the groups during and after CPB, although Hb V group showed less increase during CPB and more increase after CPB ($p=0.489$) compared with the control group.

Discussion

In hemodiluted CPB Hb V may contribute in oxygen delivery and better maintain cerebral oxygen metabolism after CPB. Thus the use of Hb V for clinical CPB prime in neonates and infants should be warranted.

# NOTE

PO−1

## Assessment of Inflammation-altered Intestinal Permeability Using Arterially Perfused Murine Jejunal Loop

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We have shown previously that arterial perfusion with hemoglobin vesicles (HbV) is superior to oxygenated Krebs solution and maintains structural and functional integrity of the isolated jejunal loop for up to 2 hours. In this study we investigated whether our model is able to detect changes in intestinal permeability induced by chronic inflammation. Balb/c mice (n=30), healthy or infected with *Helicobacter pylori* (Hp), received antibiotics or placebo for 2 weeks. Intestinal permeability was measured 2 months later. 4-5 cm segment of jejunum was selected, the terminal branch of the mesenteric artery was cannulated. The loop was perfused arterially with HbV dispensed in a modified Krebs solution with 5% albumin. Macromolecular markers $^{51}$Cr-EDTA and $^{14}$C-mannitol were administered luminally. Their concentration in the venous outflow was measured using scintillation counter and expressed as a percentage of the luminal content. Tissue samples were taken and examined for Hp, inflammation and structural damage. Data are presented as means ± SE. The perfusion with HbV resulted in a normal histology with preserved epithelium in 83.3% of experiments while in 16.7% segments we found moderate/severe epithelial damage, mostly result of prolonged surgical manipulation. Hp infection induced chronic gastritis but did not affect jejunum. Paracellular permeability for $^{51}$Cr-EDTA was higher in Hp infected mice compared to uninfected controls (0.57 ± 0.08 vs 0.27 ± 0.07, $p<0.05$) and tended to improve after eradication treatment. Membrane permeability for $^{14}$C-mannitol was also higher in Hp infected mice compared to controls (1.10 ± 0.10 vs 0.63 ± 0.15, $p<0.05$) however this did not improve after eradication treatment (1.15 ± 0.16, $p<0.05$ vs control). In conclusion, Hp infection in mice impairs intestinal epithelial barrier. Hp eradication partially improves paracellular permeability but does not affect membrane permeability. HbV-perfused intestinal loop is a valuable tool for assessing gut function in both health and disease.

# NOTE

PO—2

## Hemoglobin Encapsulation in Vesicles Retards the Reaction with NO by Intracellular Diffusion Barrier

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*[3]Kobelco Research Institute, Japan*

The reaction of NO, an endothelium-derived vasorelaxation factor, and Hb is restricted by encapsulation in RBCs to prevent vasoconstriction. This essence is applied to an artificial $O_2$ carrier, Hb-vesicles (HbV), that mimic the cellular structure of RBCs, and actually we confirmed that HbV becomes vaso-inactive in blood circulation. However, the mechanism of the NO-binding restriction is controversial; i) an extracellular diffusion barrier surrounding the RBC; ii) a RBC cytoskeletal submembrane constituting a diffusion barrier; and iii) retardation of NO-diffusion due to the viscous Hb in RBCs. To clarify the mechanism, we analyzed HbV with different intracellular Hb concentrations, $[Hb]_{in}$, and different particle sizes using stopped-flow spectrophotometry [1].

The apparent NO-binding rate constant, $k'_{NO}$, of deoxygenated HbV at $[Hb]_{in}$ = 1 g/dL was almost equal to $k_{NO}$ of molecular Hb, indicating that the lipid bilayer membrane cannot be a barrier. With increasing $[Hb]_{in}$ to 35 g/dL, $k'_{NO}$ decreased by 1/3, which was further decreased by 1/5 with enlarging particle diameter from 250 to 450 nm. For carbon monoxide (CO)-binding, which is intrinsically much slower than NO-binding, $k'_{NO}$ did not change greatly with $[Hb]_{in}$ and the particle diameter. Results obtained using diffusion simulations coupled with elementary reactions concur with these tendencies and clarify that NO is trapped rapidly by Hb from the interior surface region to the core of HbV at a higher $[Hb]_{in}$, retarding NO-diffusion toward the core of HbV. In contrast, slow CO-binding allows time for further CO-diffusion to the core. Simulations extrapolated to a larger particle (8 $\mu$m) showing retardation even for CO-binding. The obtained $k'_{NO}$ and $k'_{CO}$ yield similar values to those reported for RBCs.

In summary, the intracellular diffusion barrier is predominant owing to the intrinsically rapid NO-binding that induces a sink of NO from the interior surface to the core, retarding further NO-diffusion and binding. A high $[Hb]_{in}$, 35 g/dL, and a larger particle size are the key factors to regulate the NO-binding and to optimize artificial $O_2$ carriers comprised of phospholipid vesicles.

**Refs:** [1] Sakai et al, JBC 2008;283:1508-17; BBA 2008;1784;1441-7.

# NOTE

PO—3

## Effect of HbV as a Resuscitation Fluid in Uncontrolled Hemorrhage-shock Model: Blunt Kidney Injury Model

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【Background】 Blunt trauma is often accompanied by massive hemorrhage from solid organs' injury such as liver, kidney, pancreas. A large amount of blood can be flow out into the peritoneal space. Therefore fluid resuscitation to restore the circulation volume as well as maintenance of organ perfusion is important for these patients. Artificial oxygen carrier with colloid solution may act as volume resuscitation and oxygen infusion to tissues.
【Method】 Male Wistar rats weighing 350g were used. After general anesthesia using sevofluren, 67% hemodilution was done by serial withdrawal and infusion with HES. Then left kidney was destroyed by cutaneous manipulation. Macroscopic effect of this procedure revealed to resemble blunt rupture of the kidney. After injury, MAP decreased and Fluid resuscitation was begun when MAP reached 30mmHg. Animals were randomly divided into four groups, non-resuscitation group, 5%HSA group, HbV group.
【Results】 There are no deaths in HbV group and length of survival was good. Estimated circulation volume decreased in each group but in HbV group, decrease of circulation volume was smaller than other groups. MAP was maintained after initial drop in HbV group. Subcutaneous blood flow decreased as well as PtO2 even after resuscitation in 5%HSA group and non resuscitation group, but in HbV group, Pt O2 was maintained at the level of 15 Torr. Increase of Lactate level was inhibited in HbV group after resuscitation.
【Discussion】 Resuscitation with HbV could improve survival and circulatory indices after traumatic abdominal uncontrolled hemorrhagic injury. Stable PtO2 and inhibition of Lactate increase indicated HbV can carry enough oxygen at low blood pressure, low flow condition in peripheral tissue.

# NOTE

PO—4

## Resuscitation of Hemorrhagic Shock due to Uncontrolled Hemorrhage-effect of Hemoglobin-vesicle in Vascular Injury Model

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Uncontrolled hemorrhage is usually fatal if fluid resuscitation and adequate hemostasis cannot be achieved. Low blood pressure causes sparse perfusion and hypoxia in peripheral tissue and organ function is deteriorating in a short period. Artificial oxygen carrier may improve oxygen metabolism of organs and elongate survival.
Method: Male Wistar rats were used. After general anesthesia with sevofluren, lapalotomy was done. Uncontrolled hemorrhage was created by penetrating the aorta with 24 G needle. MAP decreased swiftly and when MAP decreased lower than 20 mmHg within a minute, resuscitation was initiated. Several resuscitation fluid such as large volume of saline, same volume of allogenic blood, same volume of 5% albumin, HbV resolved in 5%HSA was administered. MAP, HR, WBC, Ht, Hb, Lactate, Piruvate were measured. When animal died, time was recorded.
Results: Bleeding volume was 8.0+/-0.9ml in no resuscitation group, 14.3+/-2.9ml in high volume saline group, 12.6+/-4.1ml in allogeneic transfusion group, 11.8+/-4.0ml in 5%HSA group, and 9.8+/-3.7ml. In high volume saline group, constant decrease of MAP was observed even large resuscitation fluid was administered. There are significant difference in blood loss between saline group and other groups. In HbV group, blood loss was significantly smaller in amount. Change of Lactate/pyruvate ratio was smaller in allogeneic blood group and HbV group.
Discussion: In uncontrollable hemorrhage model, MAP could not be maintained by large volume of crystalloid fluid. Colloid containing fluid seemed effective for elongation of survival. Oxygen carrying capacity inhibited the increase of lactate/pyruvate ratio. HbV was thought to ameliorate organ oxygen metabolism and increase the survival time.

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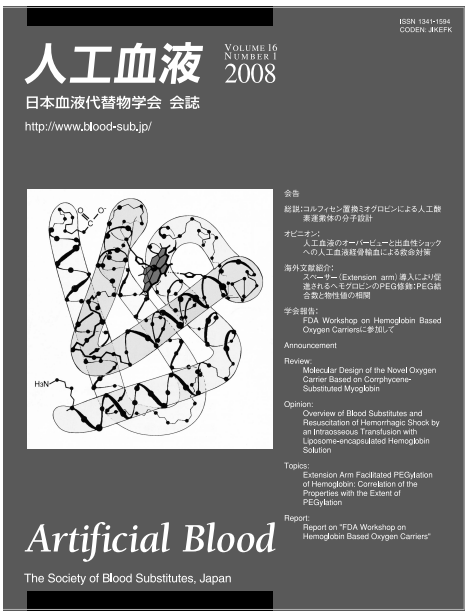


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2. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. J Am Med Assoc 2008;299:2304-2312.
3. Sakai H, Sou K, Takeoka S, Kobayashi K, Tsuchida E. In: Winslow RM, ed. Blood Substitutes. London: Academic Press (Elsevier), 2006;514-522.
4. Oxygen Infusion Project, Waseda University, Japan. http://www.waseda.jp/prj-artifblood/index-ja.html (last accessed Sept 2008)

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2）投稿論文の査読は，編集委員長が選んだ人工血液分野の研究者に依頼する．査読意見によっては，原稿の修正を求める場合がある．修正論文（Revised Manuscript）の投稿に際しては，送付状に「査読意見に対する回答」を添え，意見に対して一つ一つ回答をするとともに，修正箇所がある場合にはこれを明記する．

3）掲載決定通知の後，著者は採択論文の文章・図表のファイルを電子媒体として，指定する宛先に送付すること（使用したソフトを明記すること）．文章・表のファイル形式は，doc, txtが好ましい．図は，ppt, jpg, tiffが好ましい．

4）原稿はA4版の大きさとし，第1頁には表題，英文表題，著者名，全著者所属，英文著者名，英文著者所属，次いで連絡の取れる著者（corresponding author）の住所，英文住所を記入する．手書き原稿の場合はB5版，1行20字，20行とする．

5）「原著論文」，「総説」，「トピックス」，「オピニオン」については，第2頁以降に和文抄録，Keywords（英文で6個程度）を付け，最終頁または別紙に英文抄録を付けること．

6）投稿論文に記載の研究が公的助成を受けて実施された場合には，謝辞にその旨を記載すること．また，Conflict of Interests（例えば，論文に記載された薬品を販売する企業と著者との利害関係: 雇用，コンサルタント，研究助成，株式，特許など）があれば，これを第1頁の脚注，謝辞などに記載すること．

7）ヒトを対象とした研究結果，および動物実験の結果を掲載する場合には，各研究機関のガイドラインに従って実施したことを方法等に明記すること．

8）論文中の略語は初出の際に省略しないこと．薬品，医薬品，測定装置等は，外国語名の場合は言語のまま用い，日本語化しているものはカタカナとする．型式，販売（製造）元とその所在地も記入すること．
（例） Rhodamine B（Sigma-Aldrich, St. Louis, USA），ポリグラフシステム（LEG-1000; 日本光電工業, 東京）

9）句読点はコンマ（,）ピリオド（.）とする．

10）文中の英語に使用するフォントは，Times, Helvetica, Courier, Symbolを原則とし，英文半角小文字とする．ただし，文頭および固有名詞は大文字で書きはじめること．

11）数字はアラビア数字を使い，度量衡の単位はm, cm, mm, μm, L, mL, μL, mol, g, mg, μg, ng, pg, fg, N／10などを用いる．

12）FigureとTable：引用順にそれぞれ番号を付けること．表題，説明，図表中文字は，全て英文とすることが好ましい．本文中に挿入箇所を明記すること．Figureは直接オフセット印刷とする．Tableは編集部にて入力し原図とする．

13）文献：本文に引用した順序に番号を付け，文中では[2]，[3-5]，[1, 4-6]などとする．文献の記載法はthe Vancouver styleに従う．全著者名．論文題名．誌名　西暦発行年；巻数：頁～頁．とし，誌名の省略は医学中央雑誌またはIndex Medicus に準拠する．単行本の場合は全著者名．題名．編集者名．書名．発行地：発行書店，年号；頁～頁．の順とする．電子文献の場合は，ホームページ名．改行してアドレス（引用した西暦年月）とする．
(例)


1. 高折益彦. 人工酸素運搬体:その将来への期待. 人工血液 2007;15:90-98.
2. 橋本正晴. 単回投与毒性試験. 野村 護, 堀井郁夫, 吉田武美 編. 非臨床試験マニュアル. 東京: エルアイシー, 2001;37-48.
3. Wong NS, Chang TM. Polyhemoglobin-fibrinogen: a novel oxygen carrier with platelet-like properties in a hemodiluted setting. Artif Cells Blood Substit Immobil Biotechnol 2007;35:481-489.
4. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. J Am Med Assoc 2008;299:2304-2312.
4. Sakai H, Sou K, Takeoka S, Kobayashi K, Tsuchida E. In: Winslow RM, ed. Blood Substitutes. London: Academic Press (Elsevier), 2006;514-522.
5. 早稲田大学酸素輸液プロジェクト. http://www.waseda.jp/prj-artifblood/index-ja.html（2008年9月現在）


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c）論旨を変えないこと．執筆者は同一（順不同）であること．
d）二次掲載版のタイトル・ページに掲載される脚注には，その論文の全体もしくは一部分がすでに掲載されている旨を明記し，更に初出文献も示すこと．適切な脚注の例を以下に示す．「This article is based on a study first reported in the［...雑誌タイトル（完全な典拠情報を添えたもの）...］（訳：この論文記事は，［...］に最初に報告された研究に基づくものである）」．

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