C．結果

1．超高圧滅菌法

(1) 滅菌効果

すべての細菌、ウイルス、バチルスで、滅菌効果を認めた。

(2) に及ぼす影響

Hbに強い影響を及ぼした。変性せしめた。

Hbを変性させるので、超高圧滅菌法は本試料の滅菌には適切ではなかった。

2．β-プロピオラクトンによる滅菌法

（1）滅菌効果

処理条件が室温、BPL濃度0.05ないし0.1％での指標菌への効果を調べた（表２）。いずれも濃度及び処理時間依存的に菌数の低下を認め、0.1％では全菌種で、5時間以内にコロニーを、検出しなくなった。ただし、抵抗性はブドウ菌月良く、0.05％では5時間後も残存菌が認められた。一方、指標菌未添加のHbVに室温、BPL濃度0.05及び0.1％を添加し、24時間以上おいたものについて、外観などを調べたところ、pH低下は前述の実験同様濃度依存的に見られたが、その他の項目には大きな変化は見られなかった。

(2) Hbに及ぼす影響

結果の一覧を**表３**に示した。BPLは濃度及び温度依存的にタンパク質であるHb自体の物性に影響が見られることが分かった。特に1.0％でのpH低下は著しく、凝集沈殿まで生じた。これ未満の濃度ではpH低下と酸素親和性のパラメータ変化を伴うものの、明らかな凝集は認めなかった。

表一2：BPLの殺菌効果の確認

Solution kind	BPL 濃度	Bacteria kind	0 hour	1 hour	2 hour
Hb - V 懸濁液+BPL	0.05%	Staphylo coccus aureus	$1.3x10^6$	$3.9x10^5$	$1.8x10^4$
	0.1		$1.2x10^6$	$2.9x10^2$	$3.2x10^2$
Hb-V 懸濁液	!		$1.4x10^6$	$1.4x10^6$	$1.4x10^6$

Solution kind	BPL 濃度	Bacteria kind	0 hour	1 hour	2 hour
Hb - V 懸濁液+BPL	0.05%	Pseumonas aeruginosa	$5.2x10^6$	$1.7x10^2$	7
	0.1		$5.0x10^6$	0	0
Hb-V 懸濁液	!		$6.6x10^6$	$5.5x10^6$	$5.2x10^6$

Solution kind	BPL 濃度	Bacteria kind	0 hour	1 hour	2 hour
Hb - V 懸濁液+BPL	0.05%	Candida albicans	$1.2x10^5$	$9.4x10^3$	$1.6x10^3$
	0.1		$8.8x10^4$	0	0
Hb-V 懸濁液	!		$9.2x10^4$	$1.0x10^5$	$1.0x10^5$

Solution kind	BPL 濃度	Bacteria kind	0 hour	1 hour	2 hour
Hb - V 懸濁液+BPL	0.05%	Escherichia coli	$5.4x10^4$	$2.6x10^4$	$7.7x10^3$
	0.1		$5.5x10^4$	$2.2x10^2$	0
Hb-V 懸濁液	!		$5.2x10^4$	$6.9x10^4$	$9.9x10^4$

濃度0.1％で滅菌効果を認めた。しかもHbに影響を及ぼさなかった。使用に値する滅菌法である。

3．銀ナノ粒子による滅菌

(1) 滅菌効果

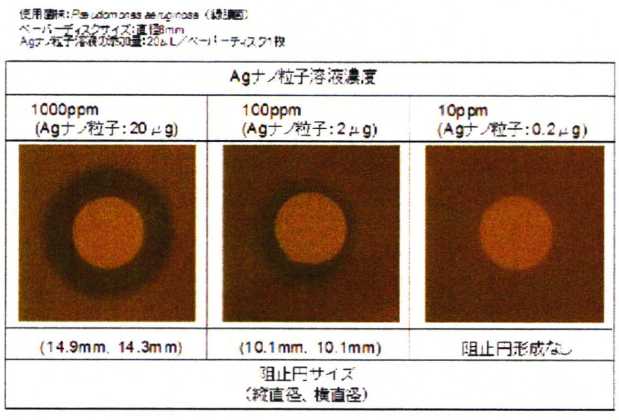
1000 ppmの濃度で明確な阻止円を認めた(図１)。

(2)Hbに対する影響

Hbには、全く影響を認めなかった。

銀ナノ粒子との接触法は、有る程度の滅菌効果を認め、Hbには影響を及ぼさなかった。使用に値する滅菌法である。

図１．銀ナノ粒子による阻止円の形成



D．考察

本研究では、HbVに対する全く新しい滅菌法は、見いだすことは出来なかった。一方、薬事法によれば、製剤に対して無菌医薬品を製造する技術としては、滅菌法と無菌操作法があり、バイオ由来(生物由来)による製剤は、無菌操作法によって、最終産物が無菌であれば、滅菌されたものと同等と見なされることになっている。

無菌操作法は、無菌医薬品を製造する場合、医薬品を最終容器に充填した後、滅菌する方法である最終滅菌法を適用しない医薬品を製造するために用いる技術であり、濾過滅菌後、又は原料段階から一連の無菌工程により、無菌医薬品を製造するために用いる方法を言う。

本操作法を用いて無菌医薬品を製造する場合は、通例、あらかじめ使用する全ての器具及び材料を滅菌した後、環境微生物数及び微粒子数が適切に管理された無菌設備内に於いて、適切な無菌操作法を用いて一定の無菌性保証水準を得られるように行う必要がある。

E．結論

成果に基づくHbVの滅菌方針として、芽胞を形

表-3 βプロピオラクトン(BPL)によるHbへの影響

処理条件 BPL終濃度	適用温度	適用時間	外観	吸収スペクトル	遠心後の沈殿物 (40,000×g、30分)	pH	p50	Hill係数
1%	−	0hr	変化なし	N.E.	N.E.	N.E.	N.E.	N.E.
	36℃	2hr	凝集塊多数認める	変化あり	N.E.	4.60	12.05	1.85
		4hr	凝集塊多数認める	N.E.	N.E.	N.E.	N.E.	N.E.
	室温	24hr	凝集塊多数認める	N.E.	N.E.	N.E.	N.E.	N.E.
	4℃	24hr	濃化・やや粘稠	変化なし	あり	4.77	7.90	1.94
0.1%	−	0hr	変化なし	変化なし	N.E.	7.52	25.56	2.39
	36℃	2hr	変化なし	変化なし	なし	6.83	18.16	2.04
		4hr	変化なし	変化なし	なし	6.81	18.46	2.00
	室温	24hr	変化なし	変化なし	なし	6.78	17.58	1.99
	4℃	24hr	変化なし	変化なし	なし	6.90	15.21	2.11
0.05%	−	0hr	変化なし	変化なし	N.E.	7.64	24.52	2.32
	36℃	2hr	変化なし	変化なし	なし	7.29	20.73	2.18
		4hr	変化なし	変化なし	なし	7.26	22.23	2.16
	室温	24hr	変化なし	変化なし	なし	7.20	21.77	2.21
	4℃	24hr	変化なし	変化なし	なし	7.29	22.03	2.18
0.01%	−	0hr	変化なし	変化なし	N.E.	7.55	24.27	2.40
	36℃	2hr	変化なし	変化なし	なし	7.40	23.66	2.22
		4hr	変化なし	変化なし	なし	7.40	23.55	2.29
	室温	24hr	変化なし	変化なし	なし	7.58	24.60	2.29
	4℃	24hr	変化なし	変化なし	なし	7.61	24.47	2.36
0%	−	0hr	−	−	なし	7.58	24.34	2.38

N.E.: Not Examined

成するBacillusの混入しないクリーンルーム内で、滅菌効果が中等度有するBPLによる滅菌を行い、全ての器具及び材料を滅菌し、無菌操作法により、本剤を製造して、薬事法に基づく無菌性保証の水準を獲得する。

F．健康危険情報

該当なし

G．研究発表

該当なし

H．知的財産権の出願。登録状況（予定を含む）

該当なし

別添4-9　　平成22年度 厚生労働科学研究費補助金（政策創薬総合研究事業）

分担研究報告書

人工赤血球の臨床応用を目指した至適投与法の策定とGMP製造技術の確立

分担課題：ヘモグロビン小胞体の心筋虚血－再灌流障害に対する保護効果

作用メカニズムの解明-4：nitroso-redox balanceと細胞内Ca^{2+}動態に関係する蛋白質の発現

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研究要旨

ラット摘出心臓をランゲンドルフ灌流し、ヘモグロビン小胞体(HbV) 30倍希釈懸濁液(Hbとして0.33 g/dL)を虚血直前に灌流すると、25分虚血後の再灌流時に心機能の回復が見られる。本実験では、前報までの検討結果を受けて、HbVがnitroso-redox balanceと細胞内Ca^{2+}動態に関係する蛋白質の心筋組織内での発現に影響を与えるか否かをwestern blot法を用いて検討した。control灌流を30分行ったcontrol群、control灌流を20分行った後にHbV 0.33 g/dLを10分間灌流したHbV群、control灌流30分を行った後に虚血25分-再灌流30分の処置を行ったischemia-reperfusion (ir)群、control灌流20分とHbV (0.33 g/dL) 灌流10分間を行った後に虚血25分-再灌流30分の処置を行ったir+HbV群の4群で心筋組織内でのSOD (Cu/Zn-SOD, Mn-SOD)、catalase、eNOS、SERCA2、calsequestrin2、phospholambanなどの蛋白質の発現を比較した。いずれの蛋白質においても、各実験群間でそれらの発現に統計的に有意な差を認めなかった。

A．研究目的

我々は、ラットの摘出心臓をランゲンドルフ灌流する方法を用いて、ヘモグロビン小胞体(HbV)が虚血-再灌流時の心機能を有意に回復させることを明らかにした(1)。そこで、この効果のメカニズムを解明するため、HbVで灌流した心筋組織中のglucose, glycogen濃度と解糖系の酵素活性を測定し、いずれの項目においてもHbVで灌流した心筋組織と対照群あるいは空球小胞体で灌流した心筋組織の間に大きな差がないことを見出した(2)。続いて、この効果がmitochondria KATP-channelを介するかどうかを検討し、このチャンネルは関係しないこと(3)、この効果がsarcolemmal KATP-channel、アデノシン受容体やプロスタノイドを経由するか否か、また心筋組織のnitroso-redox balanceを経由するか否かを検討し、この機能回復は、アデノシン受容体blocker、sarcolemmal KATP-channel blocker、PG合成酵素抑制剤では抑制されず、HbVが虚血-再灌

流後の心筋組織中のGSSGを低下させ、蛋白質のthiol残基の酸化を抑制し、NO_2 contentを低下させることから、HbVが虚血-再灌流で生じるnitroso-redox balanceの破綻を改善すること(4)、を示唆した。

本研究では、HbVが虚血-再灌流後の心機能を回復させるメカニズムの解明をさらに進めるため、nitroso-redox balanceと細胞内Ca^{2+}動態に関係する蛋白質の心筋組織内での発現をwestern blot法を用いて検討した。

B．研究方法

1．用いた試薬類

実験に用いたHbVはlot 071128 (二プロ株式会社)である。灌流液の作製には、和光純薬の特級試薬と比抵抗18.2 MΩ以下の超純水を用いた。測定する蛋白質に対する1次抗体は、BD Biosciences, Santa-Cruz Technology, Sigma, Calbiochem, Stressgen, Thermo から、2次抗体はSanta-CruzおよびCell Signalingから、購入した。

2．用いた動物、心臓灌流法および心機能の測定：

この項目での方法は前報(1-4)に述べた通りであるが、以下に概略を記載する。

生後9－12週齢のWistar系雄性ラット(Charles River Japan Inc.)を用いた。ヘパリン(ノボ・ヘパリン注1000、持田製薬) 1000 Uを腹腔内投与し、7分後に、ネンブタール60 mg/kg (ネンブタール注射液、大日本住友製薬、Na-pentobarbital 50 mg/mL溶液) を腹腔内投与して麻酔した。開腹・開胸して心臓を取り出し、直ちに氷冷したKrebs-Henseleit buffer (NaCl 116 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, glucose 11.0 mM)(以下KH-bufferと省略する)に投入して心臓の拍動を停止させた。大動脈にカニューレを挿入し、KH-bufferを用いて静水圧100 cmH_2O、37℃でランゲンドルフ灌流した。KH-bufferをはじめとする灌流液は、実験開始前から終了するまで95% O_2 +5% CO_2の混合ガスを通気し、pHを7.4に調整した。

左心室に生理食塩水を満たしたラテックス・バルーンを挿入し、圧トランスデューサー(P-50, Gould Inc.)を介して多チャンネル記録計(WS-641G, Nihon Kohden)に接続し、左室発生圧(LVDP)、左室拡張末期圧(LVEDP)、心拍数(HR)などを実験開始から終了まで連続的に記録した。バルーンの容積は、control灌流時の左室拡張末期圧(LVEDP)が0-5 mmHgになるようにした。control灌流開始時から実験終了まで、心臓を灌流して出てきた灌流液を5分毎に採取して冠灌流量(coronary flow, CF)を測定した。

3．HbVのKH-bufferへの懸濁

HbV 30倍希釈懸濁液：Hb濃度0.33 g/dL のHbV懸濁液の作製法については最初の報告書(1)で詳しく述べた。概略は以下の通りであるが、実験の必要に応じて作製する総volumeは変更した。

純水約260 mLに、832 mgのD-glucoseを溶解させた。これにKH-bufferの構成イオン成分を個別に溶解させた溶液を加え、次にHbV原液14 mLを加え、最後に純水で420 mLにメスアップした。但し、NaCl溶液のvolumeは、HbV原液14 mLが含有するNaCl (0.9%)を差し引いたものとした。

こうして作製したHbV懸濁液のHb濃度は0.33 g/dL相当となる(30倍希釈懸濁液)。

この懸濁液は、37℃に加温し、95% O_2 + 5% CO_2の混合ガスを1時間以上通気した後実験に用いた。

4．実験のプロトコール

この実験のプロトコールを**Fig. 1**に示した。各実験群の実験内容は以下の通りとした。

(1)control群 (n = 5)

control灌流を30分間行った後、圧測定用のラテックス・バルーンを引き抜き、心臓を液体窒素で冷却したアルミブロックで直ちに挟んで凍結させた。

(2)HbV 0.33 g/dL群 (n=5)：以下HbV群

20分間のcontrol灌流直後に灌流液をHbV希釈懸濁液に切り換え、同じ灌流圧で10分間灌流した。その後、control群と同様に心臓を液体窒素で冷却したアルミブロックで挟んで凍結させた。

(3)ischemia-reperfusion群 (n = 7)：以下ir群
control灌流を30分間行った後、灌流を停止させて虚血(global ischemia)を惹起した。虚血を25分間継続した後再灌流を30分間行い、その後control群と同様に心臓を液体窒素で冷却したアルミブロックで挟んで凍結させた。

(4) ir+HbV群 (n = 6)
20分間のcontrol灌流直後に灌流液をHbV希釈懸濁液に切り換え、同じ灌流圧で10分間灌流した。その後、ir群と同様に虚血25分-再灌流30分の処置を行い、control群と同様に心臓を液体窒素で冷却したアルミブロックで挟んで凍結させた。

以上の実験で凍結させた心臓は、液体窒素中で細かく粉砕して－80℃に保管した。

5. 心筋組織中の蛋白質の発現測定
(1) 心筋組織からの蛋白質の抽出：
上記の実験のcontrol群、HbV群、ir群、ir+HbV群の各5例の心臓組織を、lysis buffer (Tris-HCl 20 mM, pH 7.5, NaCl 150 mM, Na_2EDTA 1 mM, EGTA 1 mM, 1% Triton, sodium pyrophosphate 2.5 mM, β-glycerophosphate 1 mM, Na_3VO_4 1 mM, Leupeptin 1 μg/mL)で抽出し、4℃、10,000gで遠心分離した後その上清中の蛋白質をBCA法 (Thermo Scientific, Rockford, IL, USA)で測定した。残った上清は以下のwestern blottingの実験を行うまで－80℃に保管した。

(2) 抽出した蛋白質のwestern blotting：
抽出液中の蛋白質のwestern blottingは定法に従って行った。概略は以下の通り。抽出液を4％の2-mercaptoethanol を含むLaemmli sample buffer (BioRad, 161-0737)と等量混合し、heat blockを用いて95℃で5分間加熱した。SERCA2、calsequestrin 2 (以下cls2と省略)、およびphospholamban (以下plbと省略)の測定では加熱操作を行わなかった。こうして調製した処理液の蛋白質15 μg相当分を用いて12% SDS-PAGEを行った。PAGE終了後blotting buffer (Tris-glycine- SDS buffer)を用いて分離した蛋白質をゲルからPVDF膜 (BioRad, 162-0176) にblottingした(ATTO blotting装置で15V, 1.4時間)。

Fig. 1 心筋組織中のnitroso-redox balanceと細胞内Ca^{2+}動態に関係する蛋白質の発現を測定のための実験のプロトコールと実験例数

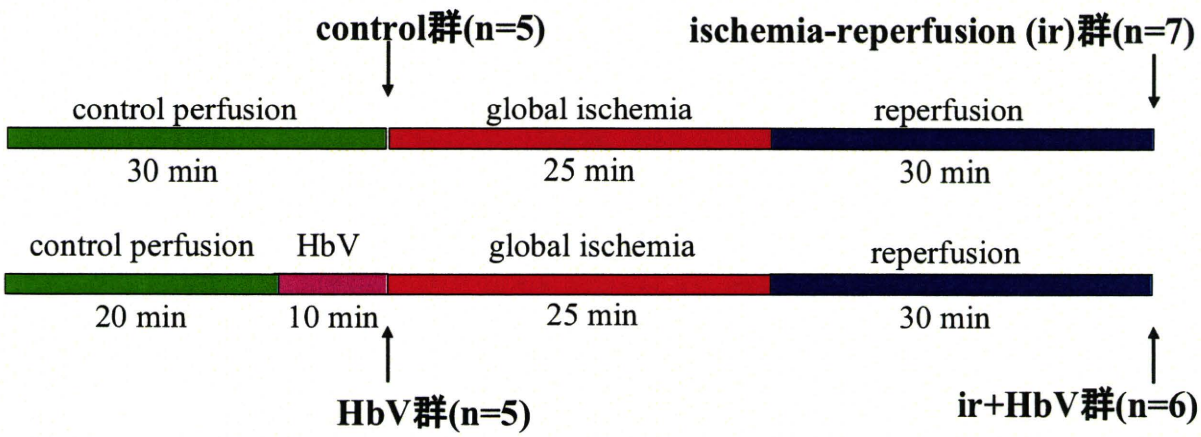
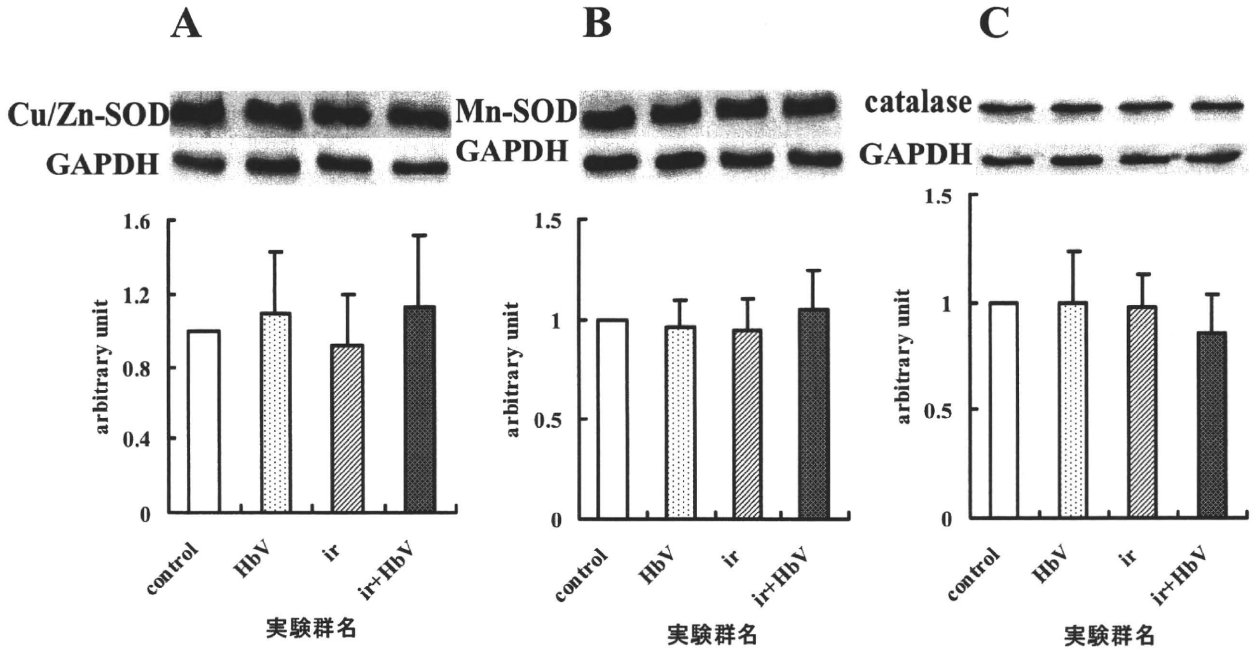


Fig. 2 心筋組織中のCu/Zn-SOD (A), Mn-SOD (B), catalase (C) の各実験群における発現



PVDF膜をblocking液(ナカライテスク, 03953-95)に1時間浸漬してblockingを行った後、10％のblocking液を含む1% Tween-20含有リン酸buffer（PBST）に溶解させた以下の1次抗体と室温で1ないし2時間反応させた。

Cu/Zn-SOD (1:1,000 dilution, Stressgen, SOD- 100)、Mn-SOD (1:11,000 dilution, Stressgen, SOD-110)、catalase (1:1,000 dilution, Calbio- chem 219010), eNOS (1:1000 dilution, BD Biosciences 610297)、SERCA2 (1:5,000 dilution, Santa-Cruz, sc-8094)、cls2 (1:2,000 dilution, Sigma C3868)、plb (1:20,000 dilution, Thermo MA3-922)、GAPDH (1:500 dilution, Santa-Cruz, sc-20357)。

反応終了後、1次抗体溶液を捨て、PBSTでPVDF膜を洗浄し、PBSTに溶解させた対応する2次抗体[anti-rabbit (Santa Cruz sc-2004), 1:25,000 dilution, anti-goat (Santa Cruz sc-2768), 1:25,000 dilution, anti-mouse (Cell Signaling #7076), 1:2,500～1:25,000 dilution]との反応を1時間室温で行わせた。2次抗体溶液を捨てた後、PBSTで洗浄し、発光検出溶液 Immobilon Western WBKLS0500 (Millipore corporation)と室温で5分間反応させ、LAS 3000 (Fuji Film)で写真撮影した。

6. データの計算と統計処理

各実験群で撮影した蛋白質の写真は、Multi Gauge Ver. 3.1(Fuji Film)を用いて計数化し、その値を対応するGAPDHの値で補正した。こうして計算した各実験群の蛋白質の個別データをそれぞれのcontrol群の値の平均値で割り算した。control群以外の計算データはcontrol群の平均値を1とした時の比例値として平均値(mean)と標準偏差(SD)で表示した。各実験群での蛋白質の値は、両側ANOVAを行った後、各実験群間の有意差をTukey多重比較法で検定した。

C．研究結果

各実験群で冠灌流量と心機能(心拍数、左室発生圧、左室拡張末期圧、±dP/dTなど)を測定したが、いずれの項目でも、これまでに報告した実験結果と大きな違いはなかったので、ここでは図示しなかった。

Fig. 2にsuperoxide anion (O_2・⁻)を代謝するSOD (Cu/Zn-SODおよびMn-SOD)と過酸化水素(H_2O_2)を還元するcatalaseの、**Fig. 3**にNOを産生するeNOSの、そして**Fig. 4**に細胞内Ca動態に関係するSERCA2, cls2およびplbの、それぞれ心筋組織内での発現結果を示した。

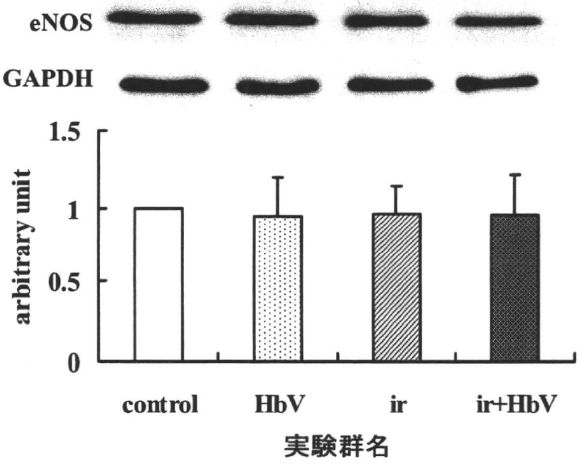
いずれの蛋白質においても、各実験群間でそれらの発現に統計的に有意な差は認められなかった。

D．考察

我々は前報(4)で、HbVが虚血-再灌流後の心筋組織中のGSSGを低下させ、蛋白質のthiol残基の酸化を抑制し、NO_2 contentを低下させることから、HbVが虚血-再灌流で生じるnitroso-redox balanceの破綻を改善することで虚血-再灌流時の心機能回復効果を発揮するだろうこと、を示唆した。

nitroso-redox balanceは、大まかに言えば、**Fig. 5**に示したように、様々な経路で合成されるsuperoxide anionとNOS酵素で合成されるNOおよびそれらが化学反応して生成するONOO・ anion (peroxynitrite)などの化合物のバランスであると考えられている(5)。これらの化合物はミトコンドリアなどの細胞内顆粒から様々な酵素に至る広範な

Fig. 3 心筋組織中のeNOS蛋白質の各実験群における発現



蛋白質で代謝制御されている。また、この中で合成されるONOO・ anionは、蛋白質のtyrosine残基の水酸基をニトロ化することや、systein残基のthiol基をニトロシル化、グルタチオニル化することで、様々な蛋白質の活性を制御する。例えば細胞内カルシウム動態を制御するryanodine receptorやSERCA蛋白質は、ONOO・ anionによってそのsystein残基のthiol基がニトロシル化される(6)ことやグルタチオニル化される(7)ことで活性が制御されていると報告されている。

Fig. 4 心筋組織中のSERCA2 (A), calsequestrin2 (B)および phospholamban (C)の各実験群における発現

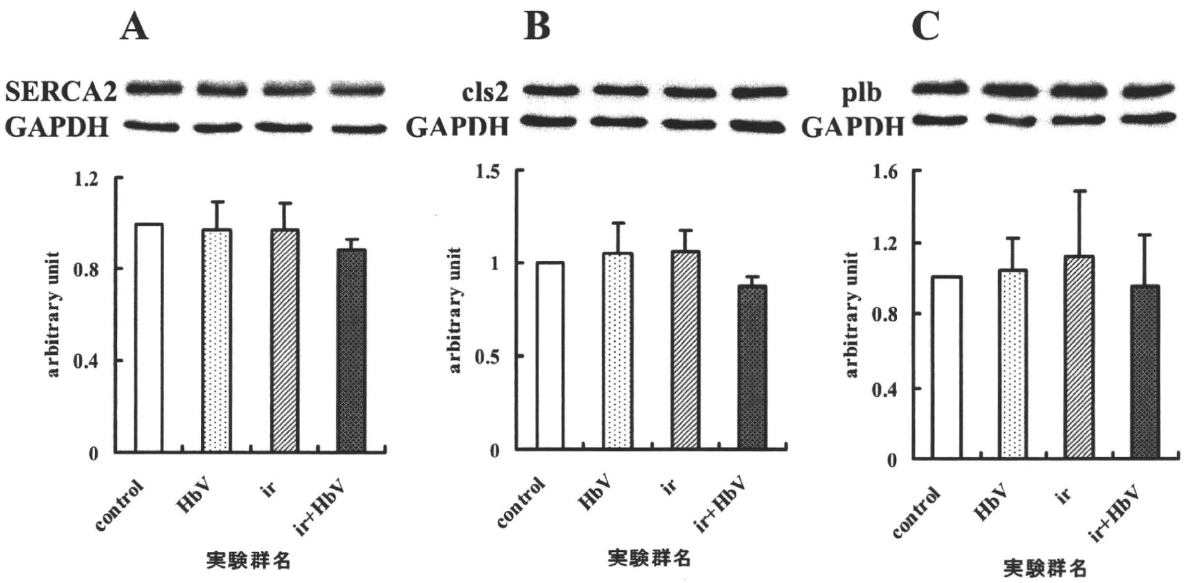
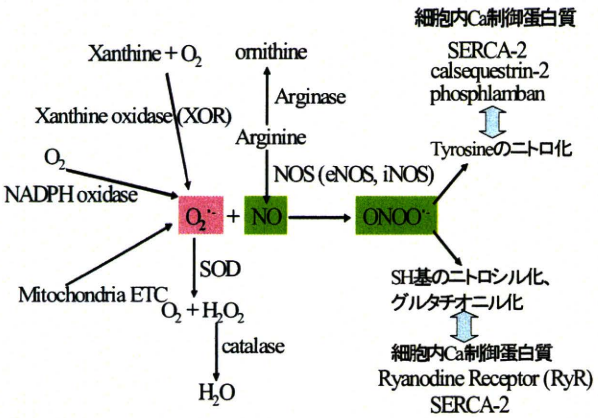


Fig. 5 nitroso-redox balanceと細胞内Ca動態を制御する蛋白質



今回の実験で我々は、まずsuperoxide anionを消去するSOD (Cu/Zn-SOD, Mn-SOD)と過酸化水素(H2O2)を還元するcatalaseの心筋組織内での発現を測定し、各実験群間でそれらの蛋白質の発現に有意の差がないことを見出した。この結果は、前報(4)で報告したこれらの酵素活性の結果と一致するものであった。さらにNOを合成するeNOS、細胞内Ca動態に関係するSERCA2, cls2およびplbについても同様の検討を行ったが、これらの蛋白質の発現においても各実験群間で有意の差を見出さなかった。今回の実験ではnitroso-redox balanceと細胞内Ca2+動態に関係する一部の蛋白質の発現を検討した。今後は、superoxide anionの合成に関わる蛋白質やiNOSの発現あるいは細胞内Ca^{2+}動態に関係する蛋白質の活性化状態なの測定をさらに進め、作用メカニズムを解明したいと考えている。

E．結論

ラット摘出心臓をランゲンドルフ灌流し、ヘモグロビン小胞体(HbV) 30倍希釈懸濁液(Hbとして0.33 g/dL)を虚血直前に灌流すると、25分虚血後の再灌流時に心機能の回復が見られる。本実験では、前報までの検討結果を受けて、HbVがnitroso- redox balanceと細胞内Ca^{2+}動態に関係する蛋白質の心筋組織内での発現に影響を与えるか否かをwestern blot法を用いて検討した。control灌流を30分行ったcontrol群、control灌流を20分行った後にHbV 0.33 g/dLを10分間灌流したHbV群、control灌流30分を行った後に虚血25分-再灌流30分の処置を行ったischemia-reperfusion (ir)群、control灌流20分とHbV (0.33 g/dL) 灌流10分間を行った後に虚血25分-再灌流30分の処置を行ったir+HbV群の4群で心筋組織内でのSOD (Cu/Zn-SOD, Mn-SOD)、catalase、eNOS、SERCA2、calsequestrin2、phospholambanなどの蛋白質の発現を比較した。いずれの蛋白質においても、各実験群間でそれらの発現に統計的に有意な差を認めなかった。

（参考文献）

1. 大鈴文孝、楠原正俊、柳田茂樹、山岸正、加藤隆一、別所基明、浜御幸．Hb小胞体の心筋虚血-再灌流障害に対する保護効果.「救急・災害医療に利用可能な人工赤血球の開発に関する研究」平成17年度 総括・分担研究報告書、pp.29-34 (2006).

2. 大鈴文孝、楠原正俊、柳田茂樹、山岸正、加藤隆一、別所基明、浜御幸．Hb小胞体の心筋虚血-再灌流障害に対する保護効果. 作用機序の解明-1：心筋組織中のglucose、glycogen濃度と解糖系酵素活性への影響「救急・災害医療に利用可能な人工赤血球の開発に関する研究」平成18年度 総括・分担研究報告書、pp. 38-43 (2007).

3. 大鈴文孝、楠原正俊、柳田茂樹、山岸正、中島淳、別所基明、浜御幸．Hb小胞体の心筋虚血-再灌流障害に対する保護効果．作用機序の解明-2：mitochondria KATP-channel活性を経由する可能性について「血液製剤安定確保のための人工酸素運搬体を用いた救急医療への応用に関する研究」平成19年度 総括・分担研究報告書、pp. 49-54 (2008).

4. 大鈴文孝、足立健、柳田茂樹、山岸正、中島淳、別所基明、浜御幸．Hb小胞体の心筋虚血-再灌流障害に対する保護効果．作用機序の解明-3：nitroso-redox balanceを経由する可能性について「人工赤血球の臨床応用を目指した至適投与法の策定

とGMP製造技術の確立」平成21年度 総括・分担研究報告書、pp. 87-96 (2010).

5. J. M. Zimmet, J. M. Hare. Nitroso-redox interactions in the cardiovascular system. *Circulation* 114, 1531-1544 (2006).

6. L. Xu, J. P. Eu, G. Meissner, J. S. Stamler. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* 279, 234-237 (1998).

7. T. Adachi, R. M. Weisblod, D. R. Pimentel, J, Ying, V. S. Sharov, C. Schoneich, R. A. Cohen. S-glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med.* 10 (11), 1200-1207 (2004).

F．健康危険情報

該当なし

G．研究発表

1．論文発表

1. K. Isoda, T. Matsuki, H. Kondo, Y. Iwakura, F. Ohsuzu. Deficiency of interleukin-1 receptor antagonist induces aortic valve disease in BALB/c mice. *Arterioscler Thromb Vasc Biol.* 30, 708 -715 (2010).

2. Y. Momiyama, R. Ohmori, ZA. Fayad, T. Kihara, N. Tanaka, R. Kato, H. Taniguchi, M. Nagata, H. Nakamura, F. Ohsuzu. Associations between plasma C-reactive protein levels and the severities of coronary and aortic atherosclerosis. *J Atheroscler Thromb.* 17, 460 -467 (2010).

3. T. Yamagishi, M. Bessho, S. Yanagida, K. Nishizawa, M. Kusuhara, F. Ohsuzu, S. Tamai. Severe, short-term food restriction improves cardiac function following ischemia/reperfusion in perfused rat hearts. *Heart Vessels.* 25, 417 -425 (2010).

4. E. Takayama, M. Bessho, K. Nishizawa, T. Yamagishi, S. Yanagida, M. Kusuhara, F. Ohsuzu, H. Nakamura. Transient increase in contraction observed during early global ischemia in Langendorff perfused rat heart is glycolysis dependent. *J. Natl. Def. Med. Coll.* 35, 184 -194 (2010).

5. Y. Momiyama, R. Ohmori, H. Uto-Kondo, N. Tanaka, R. Kato, H. Taniguchi, K. Arakawa, H. Nakamura, F. Ohsuzu. Serum resistin levels and cardiovascular events in patients undergoing percutaneous coronary intervention. J Atheroscler Thromb. 18, 108 -114 (2011).

H．知的財産権の出願。登録状況（予定を含む）

該当なし

別添 5

表　研究成果の刊行に関する一覧表

刊行書籍又は雑誌名（雑誌のときは雑誌名、巻号数、論文名）	刊行年月日	刊行書店名	執筆者名
Hepatically-metabolized and –excreted artificial oxygen carrier, hemoglobin-vesicles, can be safely used under conditions of hepatic impairment. *Toxicol. Appl. Pharmacol.* 248, 234-241 (2010)	2010 年 11 月	Elsevier	K. Taguchi, M. Miyasato, H. Ujihara, H. Watanabe, D. Kadowaki, H. Sakai, E. Tsuchida, H. Horinouchi, K. Kobayashi, T. Maruyama, M. Otagiri.
Repeated injection of high dose of hemoglobin encapsulated liposomes (hemoglobin- vesicles) induces accelerated blood clearance in a hemorrhagic shock rat model. *Drug Metab. Dispos.* 39, 484-489 (2011)	2011 年 3 月	The American Society for Pharmacology and Experimental Therapeutics	K. Taguchi, Y. Iwao, H. Watanabe, D. Kadowaki, H. Sakai, K. Kobayashi, H. Horinouchi, T. Maruyama, M. Otagiri.
Alteration in the pharmacokinetics of hemoglobin-vesicles in a rat model of chronic liver cirrhosis is associated with Kupffer cell phagocyte activity. *J. Pharmaceut. Sci.* 100, 775-783 (2011).	2011 年 2 月	Wiley	K. Taguchi, M. Miyasato, H. Watanabe, H. Sakai, H. Horinouchi, K. Kobayashi, E. Tsuchida, T. Maruyama, M. Otagiri.
Phagocytosis of liposome particles by rat splenic immature monocytes makes them transiently and highly immunosuppressive. *J. Pharmacol. Exp. Therap.* 337, 42-49 (2011).	2011 年 4 月	The American Society for Pharmacology and Experimental Therapeutics	D. Takahashi, H. Azuma, H. Sakai, K. Sou, D. Wakita, H. Abe, M. Fujihara, H. Horinouchi, K. Kobayashi, T. Nishimura, H. Ikeda.
Fluid resuscitation with hemoglobin-vesicles prevents Esherichia coli growth via complement activation in a hemorrhagic shock rat model. *J. Pharmacol. Exp. Therap.* 337, 201-208 (2011)	2011 年 4 月	The American Society for Pharmacology and Experimental Therapeutics	K. Taguchi, S. Ogaki, H. Watanabe, D. Kadowaki, H. Sakai, K. Kobayashi, H. Horinouchi, T. Maruyama, M. Otagiri.
Intravenous injection of Hb-vesicles (artificial oxygen carriers) after hemodilution with a series of plasma expanders (water-soluble biopolymers) in a rat repeated hemorrhage model. *Polymers Adv. Technol.* (in press)	印刷中	Wiley	H. Sakai, N. Miyagawa, H. Horinouchi, S. Takeoka, M. Takaori, E. Tsuchida, K. Kobayashi.
Hemoglobin vesicle as an effective blood substitute and its removal from circulating blood. *Artif. Organs* (in press)	印刷中	Wiley	H. Sakai, K. Sou, H. Horinouchi, E. Tsuchida, K. Kobayashi.
Hemoglobin-vesicle, a cellular artificial oxygen carrier, that fulfils the physiological roles of the red blood cells structure. *Adv. Exp. Med. Biol.* 662 (Oxygen Transport to Tissue XXXI) 433-438 (2010)	2010 年	Splinger	H. Sakai, K. Sou, H. Horinouchi, K. Kobayashi, E. Tsuchida.
Hemoglobin-vesicles as a cellular type hemoglobin-based oxygen carrier. In: *Chemistry and Biochemistry of Oxygen Therapeutics: from Transfusion to Artificial Blood.* (Ed. by S. Bettati and A. Mozzarelli), (in press)	印刷中	John Wiley & Sons	H. Sakai, H. Horinouchi, E. Tsuchida, K. Kobayashi.

刊行書籍又は雑誌名（雑誌のときは雑誌名、巻号数、論文名）	刊行年月日	刊行書店名	執筆者名
Superior plasma retention of a cross-linked human serum albumin dimer in nephrotic rats as a new type of plasma expander. *Drug Metab. Dispos.* (2010) 38, 2124-9.	2010 年 12 月	The American Society for Pharmacology and Experimental Therapeutics	Taguchi K, Urata Y, Anraku M, Watanabe H, Kawai K, Komatsu T, Tsuchida E, Maruyama T, Otagiri M.
Nitrosylated human serum albumin (SNO-HSA) induces apoptosis in tumor cells. *Nitric Oxide.* 2010;22(4):259-65	2010 年 5 月	Elsevier	Katayama N, Nakajou K, Ishima Y, Ikuta S, Yokoe J, Yoshida F, Suenaga A, Maruyama T, Kai T, Otagiri M.
One-step preparation of S-nitrosated human serum albumin with high biological activities. *Nitric Oxide.* (2010) 23:121-7.	2010 年 10 月	Elsevier	Ishima Y, Hiroyama S, Kragh-Hansen U, Maruyama T, Sawa T, Akaike T, Kai T, Otagiri M.
Genetically engineered mannosylated-human serum albumin as a versatile carrier for liver-selective therapeutics. *J Control Release.* (2010) 145:9-16.	2010 年 7 月	Elsevier	Hirata K, Maruyama T, Watanabe H, Maeda H, Nakajou K, Iwao Y, Ishima Y, Katsumi H, Hashida M, Otagiri M.
Pharmacokinetic properties of hemoglobin vesicles as a substitute for red blood cells. *Drug Metab Rev.* (in press)	印刷中	Informa Healthcare	Taguchi K, Maruyama T, Otagiri M.
Determination of electrolyte concentration in serum containing cellular artificial oxygen carrier (HbV). *Artif. Blood* 18, 3-8 (2010).	2010 年	日本血液代替物学会	S. Miyake, J. Takemura, M. Takaori.
Measurement of electrolyte concentrations in serum containing liposome vesicles. *Artif. Blood* 18, 91-95 (2010).	2010 年	日本血液代替物学会	S. Miyake, J. Takemura, H. Sakai, M. Takaori.
Effects of crystalloid and colloid osmotic pressure on HbV (liposome encapsulated hemoglobin) membrane. *Artif. Blood* 18, 128-133 (2010)	2010 年	日本血液代替物学会	S. Miyake, H. Sakai, M. Takaori.
Increased viscosity of hemoglobin-based oxygen carriers retards NO-binding when perfused through narrow gas-permeable tubes. *Microvasc. Res.* 81, 169-176 (2011).	2011 年 3 月	Elsevier	H. Sakai, N. Okuda, S. Takeoka, E. Tsuchida.
Severe, short-term food restriction improves cardiac function following ischemia/reperfusion in perfused rat hearts. *Heart Vessels.* 25, 417 -425 (2010).	2011 年	Splinger	T. Yamagishi, M. Bessho, S. Yanagida, K. Nishizawa, M. Kusuhara, F. Ohsuzu, S. Tamai.
Transient increase in contraction observed during early global ischemia in Langendorff perfused rat heart is glycolysis dependent. *J. Natl. Def. Med. Coll.* 35, 184 -194 (2010).	2011 年	防衛医科大学校	E. Takayama, M. Bessho, K. Nishizawa, T. Yamagishi, S. Yanagida, M. Kusuhara, F. Ohsuzu, H. Nakamura.

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Hepatically-metabolized and -excreted artificial oxygen carrier, hemoglobin vesicles, can be safely used under conditions of hepatic impairment

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ABSTRACT

The hemoglobin vesicle (HbV) is an artificial oxygen carrier in which a concentrated Hb solution is encapsulated in lipid vesicles. Our previous studies demonstrated that HbV is metabolized by the mononuclear phagocyte system, and the lipid components are excreted from the liver. It is well-known that many hepatically-metabolized and -excreted drugs show altered pharmaceutics under conditions of liver impairment, which results in adverse effects. The aim of this study was to determine whether the administration of HbV causes toxicity in rats with carbon tetrachloride induced liver cirrhosis. Changes in plasma biochemical parameters, histological staining and the pharmacokinetic distribution of HbV were evaluated after an HbV injection of the above model rats at a putative clinical dose (1400 mgHb/kg). Plasma biochemical parameters were not significantly affected, except for a transient elevation of lipase, lipid components and bilirubin, which recovered within 14 days after an HbV infusion. Negligible morphological changes were observed in the kidney, liver, spleen, lung and heart. Hemosiderin, a marker of iron accumulation in organs, was observed in the liver and spleen up to 14 days after HbV treatment, but no evidence of oxidative stress in the plasma and liver were observed. HbV is mainly distributed in the liver and spleen, and the lipid components are excreted into feces within 7 days. In conclusion, even under conditions of hepatic cirrhosis, HbV and its components exhibit the favorable metabolic and excretion profile at the putative clinical dose. These findings provide further support for the safety and effectiveness of HbV in clinical settings.

Introduction

Hemoglobin vesicles (HbV) are artificial oxygen carriers in which a concentrated hemoglobin (Hb) solution is encapsulated in a liposome, the surface of which is covered with polyethylene glycol (PEG). HbV has been shown to possess several superior characteristics to red blood cell (RBC) transfusion as follows: the absence of viral contamination (Abe et al., 2001), a long-term storage period of over 2 years at room temperature (Sakai et al., 2000), a low toxicity (Abe et al., 2006; Abe et al., 2007), no cross-matching and applications for use in veterinary care. It has also been clearly shown that HbV and RBC have comparable pharmacological effects in hemorrhagic shock model rats (Sakai et al., 2004c; Sakai et al., 2009). Based on these facts, HbV appears to have potential use as an alternative treatment of RBCs in cases of patients with massive blood loss. Since HbV functions as a substitute for RBC, an infused dose of HbV would be in excess of hundreds of times higher than that of other commercially available liposomal preparations such as AmBisome® or Doxil®. As a result this massive amount of HbV, with its associated components, including Hb, lipids and iron derived from Hb, could result in undesirable consequences in the systemic circulation and organs during its metabolism and disposition. Such an extraordinary appearance of HbV components could result in the accumulation of components in blood or organs, and could cause a variety of adverse effects as follows; (i) high levels of lipid components in the bloodstream,

Abbreviations: HbV, hemoglobin vesicle; Hb, hemoglobin; PEG, polyethylene glycol; RBC, red blood cell; MPS, mononuclear phagocyte system; CCl_4, carbon tetrachloride; rHSA, recombinant human serum albumin; WBC, white blood cell; Hct, hematocrit; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl-transferase; T-bilirubin, total bilirubin; ALP, alkaline phosphatase; BUN, urea nitrogen; CRE, creatinine; TG, triglyceride; H&E, hematoxylin/eosin; PAO, potential antioxidant; TBARS, thiobarbituric acid reactive substances; ^{3}H-HbV, ^{3}H-labeled hemoglobin vesicle.

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especially cholesterol, which are risk factors for kidney disease, arterial sclerosis and hyperlipidemia (Grone and Grone, 2008), (ii) Hb induces renal toxicity by dissociation of the tetramic Hb subunits into two dimers (Parry, 1988), (iii) free iron can trigger tissue damage induced by the Fenton reaction, which is mediated by heme (iron) (Balla et al., 2005). Since HbV would be expected to be used in cases of emergency medical treatment, it must have favorable metabolic and excretion profiles in a wide variety of situations.

Previously, we and our colleagues carried out pharmacokinetic, histological and biochemical studies of normal rats receiving of bolus HbV injection. The results obtained from these studies clearly showed that HbV is captured and metabolized by the mononuclear phagocyte system (MPS) mainly in Kupffer cells, and the lipid components are excreted from the liver *via* the bile (Sakai et al., 2001; Sakai et al., 2004a; Taguchi et al., 2009b). Similar results were also observed in a rat model of hemorrhagic shock (Sakai et al., 2009; Taguchi et al., 2009a). These findings demonstrated that HbV appears to show favorable metabolic and excretion profiles not only in normal but under hemorrhagic conditions as well, and thus HbV can be classified as a hepatically-metabolized and -excreted drug.

In the case of a clinical situation, patients with hepatic chronic cirrhosis represent a candidate for the therapeutic application of HbV, because they frequently show upper gastrointestinal tract bleeding following the rupture of portosystemic collaterals, so-called variceal bleeding. It is well-known that the pharmacokinetic and pharmacological effects of many hepatically-metabolized and excreted drugs are impaired under conditions of liver impairment (Greenfield et al., 1983; Okumura et al., 2007). Since these changes have the potential to cause serious adverse effects, some drugs are contraindicated for a person with hepatic injury. Thus, if the HbV components also show toxic or accumulative properties under conditions of liver failure, HbV may also be contraindicated for a person with liver impairment or a dose adjustment may be necessary under such conditions. Thus, before proceeding to a clinical evaluation, it is necessary to determine whether HbV can be useful as an artificial oxygen carrier under conditions of chronic liver impairment.

The purpose of this study was to perform a toxicological assessment of HbV, such as plasma biochemical parameters, histological staining, oxidative stress and the tissue distribution of HbV, after a bolus intravenous administration of HbV at a putative dose (1400 mg Hb/kg) in rats with carbon tetrachloride (CCl_4) induced liver impairment.

Materials and methods

Materials. An Hb solution was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). Pyridoxal 5'-phosphate was purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate were purchased from Nippon Fine Chemical Co. Ltd. (Osaka, Japan). 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-*N*-PEG was purchased from NOF Corp. (Tokyo, Japan). Recombinant human serum albumin (rHSA) was donated by the Nipro Corp. (Osaka, Japan). CCl_4 was purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan). Mineral oil was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan).

Preparation of HbV. HbV samples were prepared under sterile conditions as previously reported (Sakai et al., 1997). The resulting encapsulated Hb (38 g/dl) contained 14.7 mM of pyridoxal 5'-phosphate as an allosteric effector to regulate P_{50} to 25–28 torr. The lipid bilayer was comprised of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5/5/1, and 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-*N*-PEG (0.3 mol%). The average particle diameter was 250–280 nm. The HbVs were suspended in a physiological salt solution at 1000 mg Hb in 10 mL volume, and purged with N_2 prior to storage. The lipopolysaccharide content was <0.1 EU/mL.

Preparation of a rat model of chronic liver impairment induced by CCl_4. All animal experiments were undertaken in accordance with the guideline principles and procedures of Kumamoto University for the care and use of laboratory animals. All animals were maintained under conventional housing conditions, with food and water *ad libitum* in a temperature-controlled room with a 12 h dark/light cycle. The chronic liver injury rats were prepared as previously reported (Taguchi et al., 2010). In short, male Sprague–Dawley rats (48–51 g body weight; 3 weeks old; Kyudou Co. Kumamoto, Japan) were intraperitoneally injected with CCl_4 in mineral oil at a dose of 400 mg/kg (a volume of 0.45 mL/kg, CCl_4: mineral oil = 1:4) three times per week for 8 weeks.

Assessment of a rat model of chronic liver injury induced by CCl_4. One day after the last injection of CCl_4, the CCl_4 treated rats were anesthetized with ether. The blood samples were collected from the inferior vena cava. The ammonium concentration in blood was immediately determined by the indophenol reaction using an Ammonia-Test-Wako kit (Wako Pure Chemical Industries, Osaka, Japan). An aliquot of blood was collected for measurements of the numbers of RBC, white blood cells (WBC), platelet numbers and hematocrit (Hct). The remaining blood was centrifuged (3000 g, 10 min) to obtain plasma for the analysis of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GTP) and total bilirubin (T-bilirubin). All blood and plasma parameters were determined by a commercial clinical testing laboratory (SRL, Tokyo, Japan). The hepatic histopathological changes between control and CCl_4 treated rats were observed by hematoxylin/eosin (H&E) stain.

Injection of an HbV suspension. One day after the last injection of CCl_4, the CCl_4 treated rats were anesthetized with ether. Subsequently, polyethylene catheters (PE 50 tubing, outer diameter equal to 0.965 mm, and inner diameter equal to 0.58 mm; Becton Dickinson and Co., Tokyo, Japan) containing saline and heparin were introduced into the left femoral vein for use in the HbV injection. Twenty-four CCl_4 treated rats were injected with an HbV suspension (1400 mg Hb/kg) containing 5% rHSA to adjust the colloid osmotic pressure of the suspension to approximately 20 mm Hg (Sakai et al., 1997), and six CCl_4 treated rats were injected with saline containing 5% rHSA. After injection, the polyethylene catheter was removed and the skin sutured with a stitch. At 1, 3, 7 and 14 days after the HbV injection, six CCl_4 treated rats were randomly selected for collection of blood and organs. After collecting blood, the rats were euthanized by acute bleeding from the abdominal aorta and organs (kidneys, liver, spleen, lungs and heart) obtained. The organs were then weighed and resected *en bloc* for a histropathological study. The organs were fixed in 4% paraformaldehyde overnight and embedded in paraffin.

Plasma biochemical parameters. Blood samples were immediately centrifuged (3000 g, 10 min) to obtain plasma. The plasma samples were then ultracentrifuged to remove HbV (50,000 g, 30 min), because HbV interferes with some of the laboratory tests (Sakai et al., 2003). All plasma samples were stored at −80 °C until used. All plasma samples were analyzed by a commercial clinical testing laboratory (SRL, Tokyo, Japan). The analyses performed were total protein, ALT, AST, albumin, γ-GTP, alkaline phosphatase (ALP), urea nitrogen (BUN), creatinine (CRE), lipase, triglyceride (TG), phospholipids, total cholesterol, cholesterol ester, HDL-cholesterol, total bilirubin, direct bilirubin, indirect bilirubin and iron (Fe).

Histopathological examination. The organs were sectioned into 5 μm slices. Morphological changes in each organ were confirmed by hematoxylin/eosin (H&E) staining. The presence and location of hemosiderin, including free iron released by the metabolism of heme, were confirmed by Berlin blue staining. In short, the paraffin sections were deparaffinized. The deparaffinized sections were washed and then incubated with the stain solution (2 w/v% $K_4[Fe(CN)_6]$ $3H_2O$, 1 v/v% HCl) for 20 min. After washing, the sections were incubated

with a second stain solution (5 w/v% aluminum sulfate, 0.1 w/v% nuclear fast red) for 5 min.

Evaluation of oxidative stress. A 'PAO' test kit (Nikken SEIL Co., Ltd., Shizuoka, Japan) following the manufacturer's instructions, was used to evaluate the potential antioxidant (PAO), which evaluates Cu^{++} reduction on behalf of all present antioxidants, in plasma. Thiobarbituric acid reactive substances (TBARS) to measure the level of lipid peroxidation in plasma and liver were determined by means of a TBARS Assay Kit (Cayman Chemical Company, Michigan, USA) following the manufacturer's instructions. The reduced and oxidized glutathione were measured by using a GSSH/GSH quantification kit (Dojindo Molecular Technologies, Inc., MD, USA).

Pharmacokinetic study. ^{3}H-HbV, in which the lipid component (cholesterol) was radiolabeled, was prepared as previously reported (Taguchi et al., 2009b). In a typical preparation, HbV was mixed with a [1,2-^{3}H(N)]-cholesterol solution, (PerkinElmer, Yokohama, Japan) and the solution incubated for 12 h at 37 °C. Before use in pharmacokinetic experiments, ^{3}H-HbV was mixed with 5% rHSA. When ^{3}H-HbV was incubated with serum (24 h, 37 °C), the ^{3}H failed to completely dissociate from the HbV.

Twelve CCl_4 treated rats were anesthetized with ether and received a single injection of a ^{3}H-HbV suspension (1400 mg Hb/kg). Six rats were randomly selected for a plasma concentration test in which the pharmacokinetic parameters were determined. The rats were anesthetized with ether, blood samples were collected at multiple time points after the ^{3}H-HbV injection (3 min, 10 min, 30 min, 1, 3, 6, 12, 24, 48 and 72 h) and the plasma was separated by centrifugation (3000 g, 5 min). At 168 h after the injection of ^{3}H-HbV, they were euthanized and the organs (kidneys, liver, spleen, heart and lungs) were collected. Urine and feces were collected at fixed intervals in a metabolic cage. Another group of six rats were euthanized at 24 h after an injection of ^{3}H-HbV, and the plasma and organs collected. The radioactivity was determined by liquid scintillation counting (LSC-5121, Aloka, Tokyo, Japan) as previously reported (Taguchi et al., 2009b).

Data analysis. Data are shown as the means ± SD for the indicated number of animals. Significant differences among each group were determined using the two-tail unpaired Student's t-test. Pharmacokinetic analyses after HbV administration proceeded based on a two-compartment model. Pharmacokinetic parameters were calculated by fitting using MULTI, a normal least-squares program (Yamaoka et al., 1981). A probability value of $p<0.05$ was considered to indicate statistical significance.

Results

Assessment of a rat model of chronic liver impairment induced by CCl_4

To evaluate the extent of hepatic impairment of the CCl_4 treated rats, we measured the levels of AST, ALT, γ-GTP, T-bilirubin and the concentration of ammonia in blood. As a result, the AST, ALT, γ-GTP and T-bilirubin levels in plasma and the concentration of ammonium in blood were significantly increased in the CCl_4 treated rats compared to those in normal rats ($p<0.01$) (Fig. 1). From the H&E staining data, hepatic damage was clearly observed in the CCR, but not in normal rats (Fig. 1F).

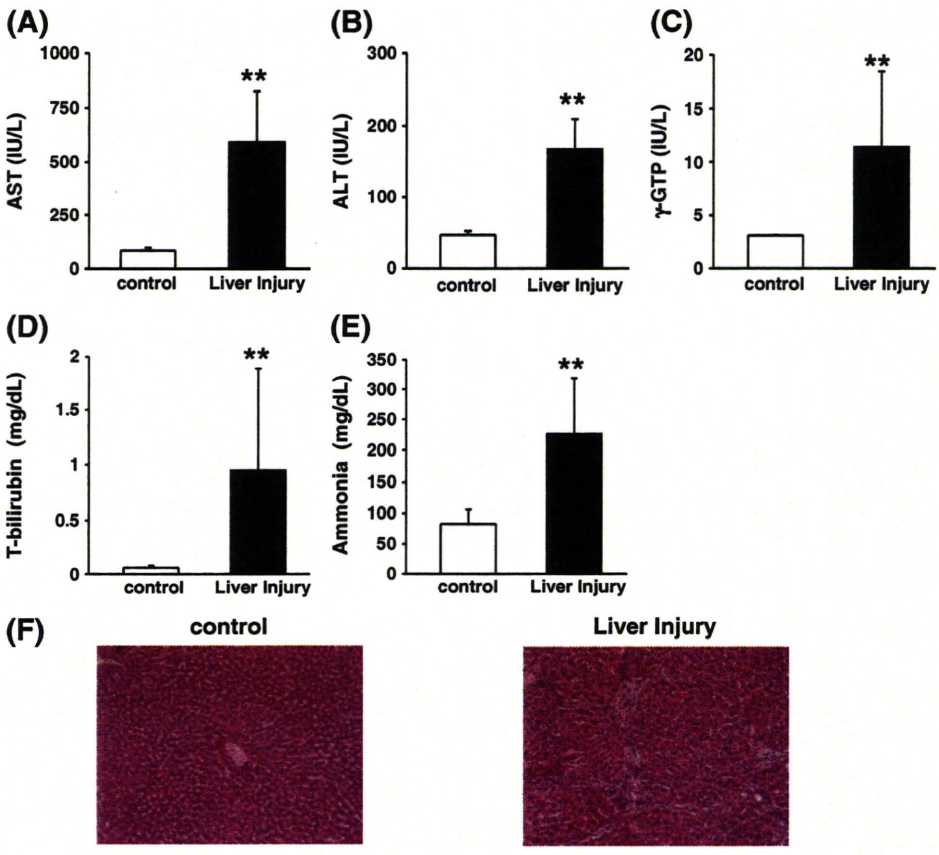

Fig. 1. Biochemical parameters (A) AST, (B) ALT, (C) γ-GTP, (D) T-bilirubin in plasma and (E) ammonia in blood in control rats (opened column) and a rat model of chronic liver injury induced by CCl_4 (closed column). (F) Light micrographs of livers stained with H&E: Control group (left) and CCl_4 treated (right) group (×100) stained with H&E. The SD rats (48–51 g body weight; 3 weeks old) were intraperitoneally injected with CCl_4 mixed with mineral oil at a dose of 400 mg/kg (a volume of 0.45 mL/kg, CCl_4: mineral oil = 1:4) three times a week for 8 weeks. One day after the last injection of CCl_4, plasma was collected and measured the biochemical parameters. The values are the mean ± SD. ($n=6$) $^{**}p<0.01$ vs. control rats.

In addition, we measured the RBC, WBC, platelet numbers and Hct in the CCl_4 treated rats. The RBC, platelet and Hct levels were not significantly changed between control and CCl_4 treated rats (RBC, 712 ± 53 and $767 \pm 55 \times 10^4/\mu L$; platelet, 67.9 ± 9.0 and $63.6 \pm 17.5 \times 10^4/\mu L$; Hct, 42.6 ± 2.7 and $44.4 \pm 0.9\%$, for control and CCl_4 treated rats, respectively), while WBC in CCl_4 treated rats was increased by approximately 3-fold compared to that in normal rats (WBC, 105 ± 29 and $281 \pm 99 \times 10^2/\mu L$; $p<0.01$, for control and CCl_4 treated rats, respectively).

In addition, the changes in organ weights, which are expressed as the percentage of organ weight relative to the body weight, were measured to examine the distribution of HbV to those organs. As a result, the organ weights (kidney, liver, lung and heart) relative to body weight did not show notable changes at each day after HbV injection (data not shown). However, the spleen weight was increased by approximately 1.2-fold at 3 days after HbV injection (100 ± 14 and $118 \pm 44\%$ of baseline, $p<0.05$, for 0 day and 3 days after HbV injection, respectively), but it recovered within 14 days after the HbV injection ($100 \pm 31\%$ of baseline).

Plasma biochemical parameters representing liver. Kidney and pancreas functions

As shown in Fig. 2, the parameters reflecting liver function (AST, ALT, γ-GTP, ALP, total protein and albumin) did not show any notable changes until 7 days after HbV injection, and some (AST, ALT, γ-GTP and ALP) were decreased slightly 14 days after the HbV infusion. However, all of these values were similar with those at 14 days after a saline injection. Although, BUN and CRE, which reflect renal function, were slightly increased until 14 days after the HbV injection, these changes were still within the normal ranges. As previously reported using normal rats (Sakai et al., 2004a), the parameter reflecting pancreatic function (lipase) was temporally increased one day after HbV, but returned to the basal level within 14 days.

Plasma biochemical parameters representing the metabolism of HbV components

The levels of TG, phospholipids, total cholesterol and cholesterol ester, which are metabolites of lipid components of HbV, were significantly increased from 1 day after HbV injection, but gradually diminished to the normal level. In fact, at 14 days after the HbV injection, the concentration of the cholesterol components (total cholesterol, cholesterol ester and HDL-cholesterol) and phospholipids were not significantly different, but only the level of TG remained higher, compared with a saline injection ($p<0.01$) (Fig. 3).

T-bilirubin, direct and indirect bilirubin, which are metabolites of internal Hb decomposition, were significantly increased 1 day after the HbV injection. However, these values gradually decreased and reached the same level as those of the saline group at 14 days. Total plasma Fe, which was also released as the result of the decomposition of Hb, did not significantly changed during the period of observation (Fig. 3).

Histological examination

In a previous study, using normal rats, hemosiderin was focused in the liver and spleen after an HbV injection (Sakai et al., 2001). Therefore, we also performed Berlin blue staining to evaluate the extent of hemosiderin decomposition in liver and spleen in CCl_4 treated rats. A

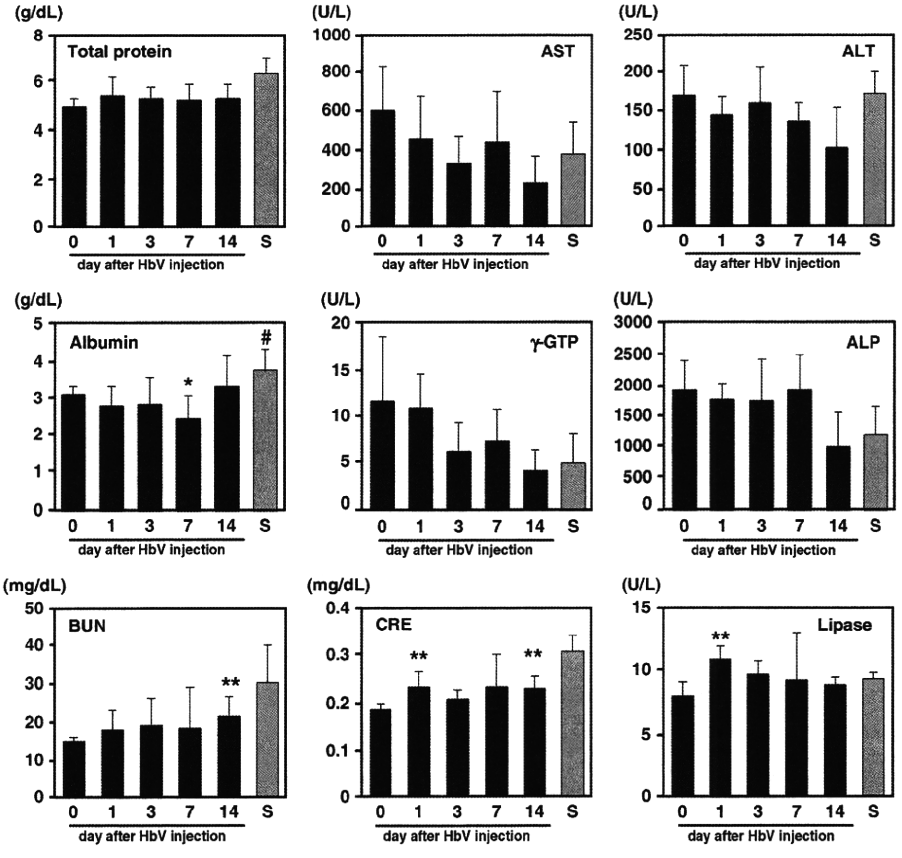

Fig. 2. Plasma biochemical parameters representing liver, kidney and pancreas function at 0, 1, 3, 7 and 14 days after HbV administration (closed column) or 14 days after a saline injection (gray column) to the rat model of chronic liver injury. The values are mean ± SD. ($n = 6$) $^*p<0.05$, $^{**}p<0.01$ vs. before HbV infusion. $^\#p<0.05$ vs. 14 days after HbV infusion. S: 14 days after saline injection.

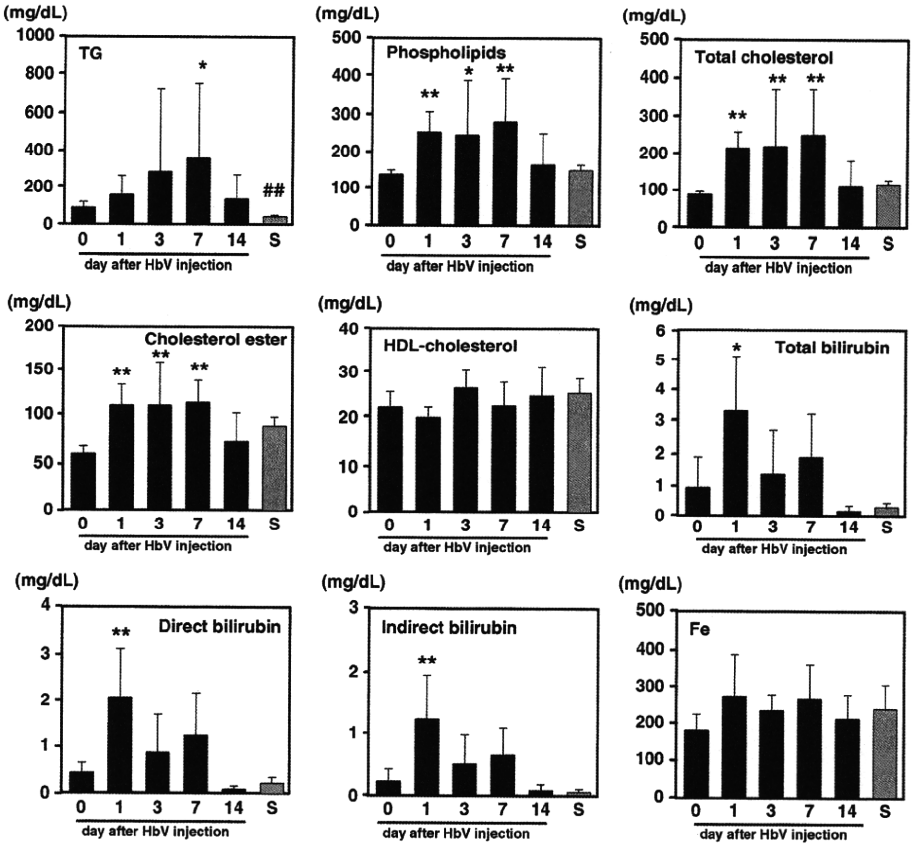

Fig. 3. Plasma biochemical parameters representing the metabolism of HbV components, such as Hb and lipid membrane, at 0, 1, 3, 7 and 14 days after the administration of HbV (closed column) or 14 days after a saline injection (gray column) to the rat model of chronic liver injury. The values are mean ± SD. (n = 6) $^{*}p<0.05$, $^{**}p<0.01$ vs. before HbV injection. $^{\#\#}p<0.01$ vs. 14 days after HbV infusion. S: 14 day after saline injection.

slight Berlin blue staining was observed in liver at days 3 and 7 (Figs. 4B and C) and a small signal was observed at 14 days after the HbV treatment (Fig. 4D). On the other hand, hemosiderin decomposition was observed in the spleen at 7 days (Fig. 4G) and at 14 days after injection of HbV (Fig. 4H). However, excessive accumulation of hemosiderin was not observed in either the liver or spleen as the result of the HbV treatment. In addition, morphological changes observed by H&E stain in kidney, liver, spleen, lung and heart were negligible after HbV injection at a dose of 1400 mg Hb/kg (data not shown).

Evaluation of oxidative stress

It has been well demonstrated that free iron enhances the production of ROS *via* the Fenton reaction, and hence, can trigger tissue damage (Balla et al., 2005). After HbV is metabolized, the heme (free iron) is released and this could induce an increase in oxidative stress. To probe this possibility further, the effect of HbV treatment on oxidative stress in plasma and liver was evaluated by measurements of TBARS and PAO. As a result, the PAO and TBARS values for plasma remained essentially unchanged as the result of HbV administration (Figs. 5A and B). In addition, the TBARS values for the liver were also determined 14 days after the HbV injection. Similar to the controls (saline treatment), a slight increase in TBARS levels in the liver was observed after the HbV infusion (0.53 ± 0.25 and 0.33 ± 0.20 μmol/L, for both the HbV and saline infusion, respectively), but the increases were not significant. To further elucidate the tissue damage by oxidative stress, the reduced and oxidized glutathione were determined. The ratio of reduced and oxidized glutathione in liver and spleen did not change during the experiments (Fig. 5C).

Pharmacokinetic study

Finally, we carried out a pharmacokinetic analysis of the lipid component of HbV in CCl_4 treated rats using ^{3}H-HbV (Fig. 6). The findings show that the approximately 10% of the HbV in plasma remained at 72 h after a bolus injection of HbV in CCl_4 treated rats. The pharmacokinetic parameters calculated using the plasma concentration curve were as follows: the half-life was 30.0 ± 4.1 h, the area under the concentration-time curve was 208 ± 51 h*% of dose/mL and plasma clearance was 0.51 ± 0.11 mL/h. Similar to normal rats, the major organs where HbV is distributed were the liver and spleen in CCl_4 treated rats (Taguchi et al., 2009b), and the maximum hepatic and splenic distributions of ^{3}H-HbVs in CCl_4 treated rats were observed at 24 h after HbV injection (25.6 ± 9.2, 14.7 ± 3.5% of the injected dose, for the liver and spleen, respectively). The majority of the ^{3}H activity in CCl_4 treated rats was excreted into feces, as previously reported for normal rats (Taguchi et al., 2009b). These data indicate that the excretion pathway of lipid components derived from HbV was essentially the same for normal and CCl_4 treated rats. The findings also confirmed that the ^{3}H radioactivity had nearly completely disappeared from the plasma and each organ 168 h after the HbV injection, and simultaneously nearly 100% of ^{3}H radioactivity had been excreted.

Discussion

As described in the introduction, HbV is one of the hepatically-metabolized and -excreted drugs. In order to avoid unpredicted or unexpected adverse effects, some of these drugs are contraindicated for a subject with hepatic impairment due to the accumulation of the drug

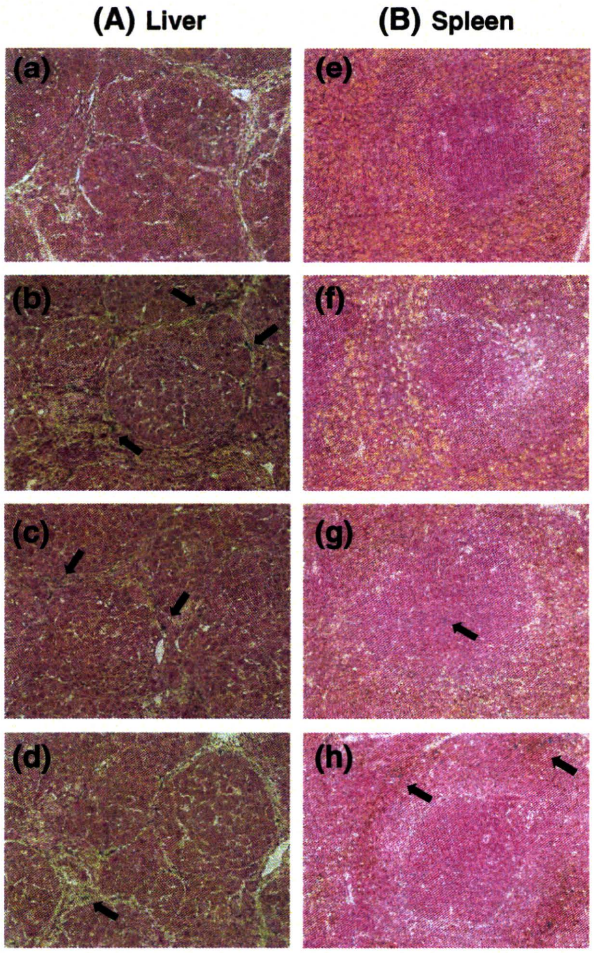

Fig. 4. Hemosiderin in liver (A) and spleen (B) in CCl_4 treated rat at 1 (a, e), 3 (b, f), 7 (c, g) and 14 (d, h) days after HbV injection stained with Berlin blue (×100). Berlin blue staining was performed to determine if hemosiderin was present. Slight hemosiderin deposition was observed in the liver at 3, 7 14 days after injection of HbV (arrow), and was also observed in the spleen at 7 and 14 days after injection of HbV (arrow).

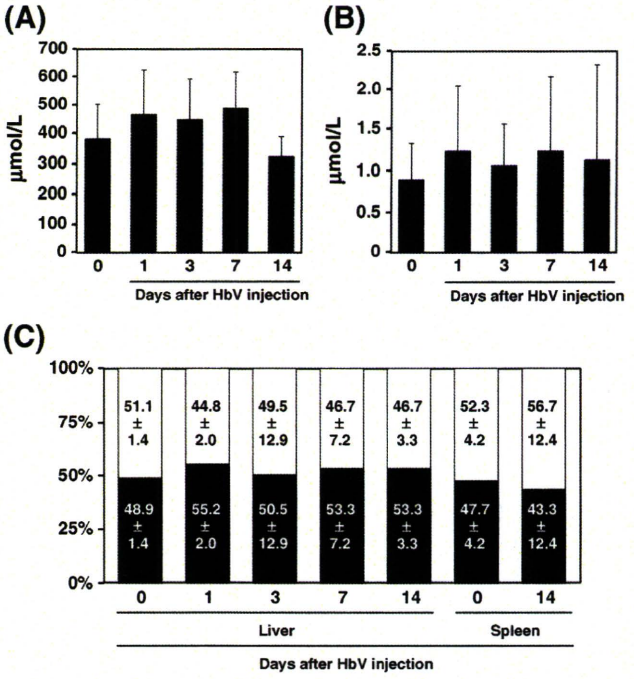

Fig. 5. Changes in the oxidative stress markers at 0, 1, 3, 7 and 14 days after HbV injection. The oxidative stress was determined by (A) potential antioxidants in plasma (PAO), (B) thiobarbituric acid reactive substances (TBARS) in plasma and (C) reduced (white) and oxidized (black) glutathione in liver and spleen. The values are the mean ± SD. (n = 6), $^{*}p<0.05$, $^{**}p<0.01$ vs. before HbV injection (0 day).

in the body. In clinical situations, a massive dose of HbV would be administered to patients, with not only hemorrhagic shock, but also hepatic chronic cirrhosis. Therefore, if HbV were to be in widespread clinical use as an RBC substitute, both therapeutic effectiveness and the safety profile in chronic liver impairment would need to be verified. In this study, we conducted the toxicological evaluation of HbV and its components, including Hb, iron derived from the heme and lipids which have the potential to be toxic in the rat model of chronic liver impairment. The results clearly demonstrate that HbV can be used safely as normal rats based on following reasons.

In the past, the perfluorocarbons have been excluded as possible candidates for artificial oxygen carriers because of the induction of chronic pneumonitis. This is due to the fact that perfluorocarbons are excreted inefficiently and accumulated in the lung, a condition that persists for more than 1 year, because perfluorocarbons was expired in a gaseous form along with the respiratory air (Nose, 2004). Thus, the long-term accumulation of an artificial oxygen carrier preparation or its components in the body must be minimized. The present study clearly showed that the half-life of HbV in the rat model of liver impairment was approximately 30 h, nearly same as that in normal rats. This suggests that HbV is not likely to accumulate in the body, even under conditions of liver failure. Since the retention in circulation is one of the therapeutic evaluation of HbV, we previously predicted that the half-life of HbV at a dose of 1400 mg Hb/kg in humans appears to be 3–4 days to use allometric equation (Taguchi et al., 2009a), and this half-life indicates that it could be used as a temporary oxygen carrier until a blood transfusion is administered or until autologous blood is recovered after a massive hemorrhage. Therefore, it would be also expected that HbV would likely possess good retention characteristics in the circulation, similar to normal conditions, even in cases of hepatic impairment.

It is well-known that the Hb derived from hemolysis and cell-free Hb based oxygen carriers (HBOCs) can induce renal and heart toxicity.

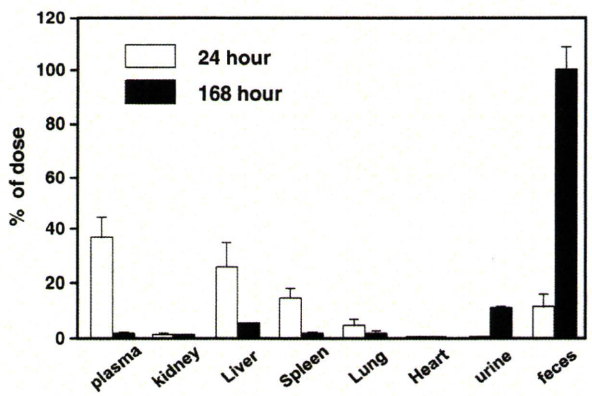

Fig. 6. The radioactivity (% of dose) in the plasma, kidney, liver, spleen, lung, heart, urine and feces at 24 (opened column) and 168 (closed column) hour after ^{3}H-HbV injection. The CCl_4 treated rats were given a single injection of ^{3}H-HbV suspension (1400 mg Hb/kg). At 24 and 168 h after the injection of ^{3}H-HbV, the rats were euthanized and organs (plasma, kidneys, liver, spleen, heart and lungs) were collected. Urine and feces were collected at fixed intervals in a metabolic cage. The values are the mean ± SD. (n = 6).

In fact, hemolysis causes renal toxicity by dissociation of tetramic Hb subunits into two dimmers, extravasaion, and precipitation in tubules (Parry, 1988). On the other hand, some of cell-free HBOCs had been excluded from proceeding to the clinical trial stage, due to the appearance of unexpected serious side effects induced by Hb. For example, diaspirin crosslinked Hb leads to the development of myocardial lesions by decreasing nitric oxide levels 24–48 h after a single topload infusion (Burhop et al., 2004), causing serious adverse effects, especially myocardial infarctions in humans (Natanson et al., 2008). Therefore, during the preclinical evaluation of HbV, the possibility of the induction of the heart and renal toxicity needs to be addressed. The findings herein indicate minimal morphological changes in heart and kidney tissue, and only negligible changes in CRE and BUN after an HbV injection at a dose of 1400 mg Hb/kg in the case of CCl_4 treated rats. This could be due to the characteristics of HbV in which its structure is maintained intact in the circulation and Hb derived from HbV was completely degraded by MPS. In fact, several results observed in this study and previous reports support these conclusions: (i) total, direct and indirect bilirubin, which were released during the metabolism of Hb, were increased from 1 day after HbV infusion, (ii) neither protein urea nor hemoglobinuria were detected in the urine in this study (data not shown), (iii) HbV is circulated in the form of stable HbV in the blood circulation until metabolized by MPS (Taguchi et al., 2009b).

Similarly, the heme (iron) is also released as a result of the metabolism of Hb, which is probably caused by the inducible form of heme oxygenase-1 in MPS in liver and spleen (Braggins et al., 1986). Generally, iron derived from heme is stored in the ferritin molecule, while overloaded iron is present as hemosiderin (Selden et al., 1980). In this study, the total Fe level in plasma was not notably changed during the period of our observations, in contrast, hemosiderin deposition was confirmed in the liver even at 14 days after HbV injection. Since hemosiderin can also release iron molecules, such released free iron could potentially induce hydroxyl radical production, and subsequently to succeed lipid peroxidation (Grady et al., 1989). However, the risk of ROS induction, as mentioned above, can be excluded in the case of HbV, because several ROS markers, such as PAO and TBARS, in the plasma did not increase during the experimental period. In addition, the TBARS level and the ratio of reduced and oxidized glutathione at 14 days after HbV injection in the liver were the same after the saline infusion. In fact, it was clearly demonstrated that the amount of iron released from hemosiderin is substantially less than that from ferritin (O'Connell and Peters, 1987). These results indicate that excess iron (heme) derived from HbV in liver impairment is likely stored as an inert form (hemosiderin) as well as that from the transfusion of RBCs and other Hb based oxygen carriers (Lenz et al., 1991; Sakai et al., 2004b).

We previously reported that the outer lipid components, especially cholesterol, were mainly eliminated to the feces *via* biliary excretion in a normal rat study (Taguchi et al., 2009b). Therefore, the possibility that these lipid components could accumulate in the body for a long period under chronic liver impairment conditions cannot be completely ignored. However, our data clearly showed that the ^{3}H-cholesterol in HbV showed a similar tissue distribution profile, mainly to the liver and spleen, as under normal conditions, and is subsequently excreted into feces within 168 h. In addition, TG, phospholipids, total cholesterol and cholesterol ester, which relate to the metabolic routes of the lipid components of HbV, were temporarily increased at 1 day after HbV infusion, but returned to normal levels within 14 days after the HbV infusion. These results indicate that the lipid components derived from HbV did not accumulate for a long period, even under hypo-metabolized and -excreted conditions.

In the present study, lipase, which reflects pancreatic function, was significantly increased on 1 day after HbV infusion. At glance, this change could be due to the damage of pancreases by HbV. However, when the pancreas is damaged, lipase levels become dramatically elevated, increasing by more than 50-fold, compared to the present data (Hofbauer et al., 1996). Thus, the small elevation of lipase level observed here can likely be attributed to the induction of pancreatic enzymes, as the result of the presence of a large amount of lipids from liposomes rather than damage caused by HbV. In fact, it has also been reported that the injection of HbV into the normal rats and liposome amphotericin B to humans also results in an increase in serum lipase activity (Stuecklin-Utsch et al., 2002; Sakai et al., 2004a).

Based on those findings, it appears likely that HbV could be safely used as an artificial oxygen carrier as an RBC substitute, even in cases of liver impairment. However, it is premature to absolutely conclude this based on the present data that was conducted under limited condition. First, in this study, we only examined the histological and biochemical parameters in a rat model of chronic liver impairment induced by CCl_4. Since the pathology of liver failure is not simple and differences in the extent of liver damage exist, such as fibrosis and injury, it will be necessary to investigate this aspect of the issue using other liver impairment animal models, for example, concanavalin A and acetaminophen induced models. Second, a previous study showed that a single high dose of HbV did not induce any change in arteriolar or venular diameters after an HbV infusion (Cabrales et al., 2005). However, Hb molecules can trigger hypertension derived by scavenging endothelium-derived nitric oxide (synthesized by NOS3) (Yu et al., 2008). In this study, changes in blood pressure, arteriolar and venular diameters were not monitored. Further study will be needed to elucidate these issues.

Recently, Zapletal et al. showed that acellular type hemoglobin-based oxygen carrier (HBOC-201) attenuated the microvascular dysfunction and improved the tissue oxygenation following the ischemic reperfusion injury in liver (Zapletal et al., 2009). Furthermore, it was reported that acellular type HBOC was useful for prehospital resuscitation with uncontrolled hemorrhage due to liver injury and hepatic cirrhosis (Ortiz et al., 2000; Arnaud et al., 2008). These results suggest that HbV and acellular type HBOC are potential candidates for alternative treatment of RBCs during liver transplantation, hepatic injury and bleeding induced by hepatic cirrhosis.

In conclusion, we demonstrate herein that HbV and its components perform favorably, in terms of metabolism and excretion, under conditions of the approximate clinical applications. These characteristics of HbV make it desirable as an alternative blood substitute, because HbV has been proposed to be used in all types of patients in multiple emergency situations including liver failure. Thus, the findings reported here provide further evidence in support of the safety and effectiveness of HbV as an oxygen carrier.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

Abe, H., Ikebuchi, K., Hirayama, J., Fujihara, M., Takeoka, S., Sakai, H., Tsuchida, E., Ikeda, H., 2001. Virus inactivation in hemoglobin solution by heat treatment. Artif. Cells Blood Substit. Immobil. Biotechnol. 29, 381–388.

Abe, H., Fujihara, M., Azuma, H., Ikeda, H., Ikebuchi, K., Takeoka, S., Tsuchida, E., Harashima, H., 2006. Interaction of hemoglobin vesicles, a cellular-type artificial oxygen carrier, with human plasma: effects on coagulation, kallikrein-kinin, and complement systems. Artif. Cells Blood Substit. Immobil. Biotechnol. 34, 1–10.

Abe, H., Azuma, H., Yamaguchi, M., Fujihara, M., Ikeda, H., Sakai, H., Takeoka, S., Tsuchida, E., 2007. Effects of hemoglobin vesicles, a liposomal artificial oxygen carrier, on hematological responses, complement and anaphylactic reactions in rats. Artif. Cells Blood Substit. Immobil. Biotechnol. 35, 157–172.

Arnaud, F., Hammett, M., Philbin, N., Scultetus, A., McCarron, R., Freilich, D., 2008. Hematologic effects of recombinant factor VIIa combined with hemoglobin-based oxygen carrier-201 for prehospital resuscitation of swine with severe uncontrolled hemorrhage due to liver injury. Blood Coagul. Fibrinolysis 19, 669–677.