死戦期を伴う心停止ドナーのグラフト肝に生じる障害機序の解明とその予防法の確立

（臨床応用を目的としたサイトカインおよび転写因子制御法の開発）

（課題番号　13557103）

平成13年度～平成15年度科学研究費補助金（基盤研究(B)(2)）
研究成果報告書

平成15年３月

研究代表者　大河内　信　弘

（筑波大学臨床医学系教授）

目　次

ページ
1. はしがき・・・・・・・・・・・・・・・・・1
2. 研究組織・・・・・・・・・・・・・・・・・1
3. 研究経費・・・・・・・・・・・・・・・・・1
4. 研究発表・・・・・・・・・・・・・・・・・2
5. 研究背景・・・・・・・・・・・・・・・・・5
6. 研究目的・・・・・・・・・・・・・・・・・6
7. 研究の独創性・・・・・・・・・・・・・・・7
8. 研究の意義・・・・・・・・・・・・・・・・8
9. 研究の位置づけ・・・・・・・・・・・・・・9
10. 申請書における研究計画・・・・・・ 11
11. 研究結果の要旨・・・・・・・・・・・ 14
12. 行った研究の方法・結果・考察
（1）課題1及び2：死戦期のを伴った心臓死ドナー肝グラフトにおける冷保存-再灌流障害発生機序の分子レベルでの解析（肝 Kupffer 細胞の果たす役割を中心に）
・・・・・・・・・・・・・ 17
（2）課題3：死戦期を伴う心停止ドナー肝の冷保存・再潅流障害に対する薬剤の投与効果の検討
・・・・・・・・・・・・・ 29
（3）課題4：臨床応用を前提とした小動物を用いた死戦期を伴う心臓死ドナー肝の障害に対する薬剤の効果の検討（ラットを用いた移植実験）
・・・・・・・・・・・・・ 72
（4）課題4：臨床応用を前提とした大動物を用いた死戦期を伴う心臓死ドナー肝の障害に対する薬剤の効果の検討（豚を用いた移植実験）
・・・・・・・・・・・・・ 82

はしがき

諸外国において脳死肝移植は、すでに難治性末期肝障害に対する治療法として確立されている。しかしながら、いずれの国においても移植数の増加に伴いドナー不足のため患者のニーズに十分に応えられない状態であり、グラフトをいかに増やすかが重要な課題となっている。一方、本邦においても脳死移植法の制定により脳死肝移植を行う環境が整いつつあるが、現時点で未だ不十分であり十数例の脳死肝移植が行われたにすぎず、今後もグラフトの提供数は非常に少ないと予想されるため、正常な体にメスを入れる生体肝移植が主流を成すと予測される。また、脳死ドナーから肝摘出がされた場合も、本邦におけるドナーはその多くがさまざまな死戦期を被っており、グラフトの状態は決して満足できるものでない。そこで、今後肝移植を医療として普及・定着させるためには、一つには提供者の増加が必要不可欠と考えられるが、死線期を被ったドナーからでも安全に肝移植が行えるようにすることが重要と考えられる。

本科学研究費により死線期を被った究極的な状態である心臓死ドナーからの肝移植の可能性の追求、つまり死線期を被った肝を移植した場合の障害発生機序の解明と、それに基づくグラフト viability の保護、改善法の開発に関して研究を行い、本書に述べる将来臨床応用が可能と考えられる有用な結果が得られたので報告する。

研究組織

研究代表者：大河内信弘　（筑波大学臨床医学系　教授）
研究分担者：藤盛　啓成　（東北大学大学院医学系研究科　助教授）
研究分担者：土井　秀之　（東北大学医学部付属病院　助教授）
研究分担者：川岸　直樹　（東北大学大学部付属病院　助手）
研究分担者：小山田　尚　（東北大学大学部付属病院　助手）

交付決定額（配分額）

	直接経費	間接経費	合計
平成１３年度	10,100 千円	0 円	10,100 千円
平成１２年度	1,800 千円	0 円	1,800 千円
総計	11,900 千円	0 円	11,900 千円

研究発表

(1) 学会誌等

1) K Oikawa, N Ohkohchi, M Satoh, et al: Elimination of Kupffer cells Suppresses activation of nuclear factor kappa B and production of cytokines and eicosanoids in non-heart-beating donors. Transplant Proc, 33:839-840, 2001

2) K Oikawa, N Ohkohchi, M Satoh, et al: Kupffer cells play an important role in the cytokine production and activation of nuclear factors of liver grafts from non-heart-beating donor. Transplant International, 15:397-405, 2002

3) S Miyagi, N Ohkohchi, K Oikawa, et al: The effect of FR167653 on warm ischemic/reperfusion injury of the liver graft from NHBD. Proceeding of the Congress of the European Society for Surgical Research, pp 247-251, 2002

4) N Ohkohchi, K Oikawa, S Tsukamoto, et al: Suppression of Kupffer cell function and administration of protease inhibitor are the keys for successful liver Tx in non-heart beating donor. Proceeding of the Congress of the European Society for Surgical Research, pp399-403, 2002

5) M Sato, N Ohkohchi, S Tsukamoto, et al: New strategy for liver transplantation from non-heart-beating donors. Transplantation Proceedings, 34:2608-2609, 2002

6) N Ohkohchi: Mechanisms of preservation and ischemic/reperfusion injury in liver transplantation. Proceedings, 34:2670-2673, 2002

7) M Sato, N Ohkohchi, S Tsukamoto, et al: Successful liver transplantation from agonal non-heart beating donor in pig. Transplant International, 16:100-107, 2003,

(2)口頭発表

1) N Ohkohchi: Mechanisms and prevention of ischemic/reperfusion injury in liver transplantation. 6th Congress of the International Society for Organ Sharing. 2001年7月25日

2) M Sato, N Ohkohchi, S Tsukamoto, et al: New strategy for liver trsnsplantation from non-heart-beating donor. 6th Congress of the International Society for Organ Sharing. 2001年7月25日

3) N Ohkohchi, K Oikawa, S Tsukamoto, et al: Suppression of Kupffer cell activity is a key for success of non-heart-beating donor. A Transplant Odyssey 2001, ２００１年８月２１日

4) N Ohkohchi, T Hirano, S Satomi: Reperfusion injury in living donor transplantation. 2nd Japan-Korea Liver Transplantation Symposium, ２００１年９月２３日

5) M Sato, N Ohkohchi, T Orii et al: Successful liver transplantation from agonal non-heart beating donor. 10th Congress of European Society for Organ Transplantation. ２００１年１０月１０日

6) 宮城重人、大河内信弘、及川公正、他：肝温阻血障害障害に対するTNFa及びIL1b抑制剤の効果-心停止ドナー肝移植を目指して-。第１０２回日本外科学会、２００２年4月１３日

7) 佐藤雅英、大河内信弘、塚本茂樹、他：心停止ドナーからの肝移植における prostaglandinおよびサイトカイン産生抑制剤投与効果の検討。第１０２回日本外科学会、２００２年4月１３日

8) S Miyagi, N Ohkohchi, K Oikawa, et al: The effect of FR167653 on warm ischemic/reperfusion injury of the liver graft from NHBD. 37th Congress of of the European Society for Surgical Research, 2002年5月２３日

9) N Ohkohchi, K Oikawa, S Tsukamoto, et al: Suppression of Kupffer cell function and administration of protease inhibitor are the keys for successful

liver Tx in non-heart beating donor. 37th Congress of the European Society for Surgical Research, 2002年5月23日

10) S Miyagi, N Ohkohchi, K Oikawa, *et al*: The effect of the new cytokine suppressive drug of FR162653 and the protease inhibitor rinse on warm ischemia/reperfusion injury of the liver graft from non-heart-beating donor toward success for cadaveric liver transplantation from NHBD. 21th International Congress of Transplantation Society, 2002年8月27日

11) N Ohkohchi, K Oikawa, S Tsukamoto, et al: Suppression of Kupffer cell function and administration of protease inhibitor are keys for successful liver Tx in non-heart-beating donor. 64th Annual Meeting of Society of University Surgeons, 2003年2月14日

(3)出版物
無し

研究背景

諸外国において脳死肝移植は、すでに難治性末期肝障害に対する治療法として確立されている。しかしながら、いずれの国においても移植数の増加に伴いドナー不足のため患者のニーズに十分に応えられない状態であり、グラフトをいかに増やすかが重要な課題となっている。一方、本邦においても脳死移植法の制定により脳死肝移植を行う環境が整いつつあるが、現時点で未だ十数例の脳死移植が行われたにすぎず、今後もグラフトの提供数は非常に少ないと予想される。また、脳死ドナーから肝摘出がされた場合も、ドナーは死戦期を被っており、グラフトの状態は決して満足できるものでない。そこで、今後肝移植を医療として普及・定着させるためには、心臓死（心停止と同義語）ドナーからの肝移植の可能性の追求、つまり死線期を被った肝を移植した場合の障害発生機序の解明と、それに基づくグラフト viability の保護、改善法の開発が重要かつ急務な課題である。

研究目的

心臓死肝移植は、primary graft nonfunction (PNG) が約50%と高率に発生するため、現段階では臨床では不適切とされている。心臓死ドナーからのグラフトと、脳死肝移植のドナーのグラフトとの明らかな違いは、死戦期を経る際の肝臓が被る温阻血障害の有無である。これまでの心臓死モデルは、 薬物による瞬間的心停止、大動脈の cross clump による機械的心拍出停止である。我々は呼吸停止により心臓死を誘導するモデルを用いて肝移植を行ったところ、移植成績は諸家の報告より有意に悪かった(Tsukamoto et al : Transplantation,1999)。心臓死肝移植の検討に際しては、低血圧、低酸素血症、アシドーシス等に暴露されるいわゆる死線期の影響を分子レベルで解析する必要がある(Ohkohchi et al. Transplant. Proc. in press)。一般的に肝実質細胞は温阻血障害を受けやすく、冷保存・再潅流障害により類洞内皮が障害を受けるとされる。当教室ではこれまでに、心臓死ドナー肝であっても移植再潅流後の血流、すなわち微少循環を維持できれば肝実質細胞機能は改善することを明らかにしている。本研究の目的は以上の結果をふまえ、死戦期を被った肝の阻血再潅流障害の発生機序を分子レベルで解析し、さらに臨床応用を見据えたその予防法の確立を行うことである。具体的には(A) 障害発生機序の分子レベルでの解析；死戦期を被った肝の虚血再潅流障害における、i) NF-kappaB, AP-1 などの転写因子の活性変化、MAP キナーゼの活性変化、c-jun キナーゼ（ JNK ）や MAP キナーゼファミリーの上流に位置する MEKK-1 やホモログの NIK などの核内転写因子およびキナーゼ群の活性変化、 ii) NF-kappaB により誘導されるサイトカイン（ IL-1, IL-2, IL-6, GM-CSF, TNF など)、ケモカイン、接着分子（ E-selectin, ICAM-1, VCAM-1 など ）、NOS 、Fas ligand などの発現変化、iii) 免疫応答や炎症反応の際活性化されるアラキドン酸代謝の key enzyme であるホスホリパーゼ A2 活性変化を検討し、総合的に阻血再潅流障害の発生機序を分子レベルで解明する。(B) 死戦期を被った肝の虚血再潅流障害の予防法の開発；i)mRNA による各種転写因子の制御、ii) 薬剤による Kupffer 細胞機能（サイトカイン、ケモカイン分泌）の抑制効果、iii) protease-inhibitor の投与による kinase activity の抑制や、heat shock protein 誘導による免疫応答や炎症反応の抑制効果について大動物を用いて検討し、最終的には臨床応用を計る。

本研究の学術的な特色・独創的な点

1.　死戦期を有する心臓死ドナーからの肝移植を可能にすることの意義：
多くの温阻血に関する研究で用いられている心臓死ドナーのモデルは塩化カリウムで心臓死を誘導しているものが多く、このようなモデルは低酸素、pH の低下、低血圧、腸管のうっ血といった、いわゆる、死戦期を経ていない。しかし実際の臨床の場において死戦期を経ない心臓死というのは極めて稀である。死戦期には腸管でのサイトカイン産生が亢進し、それが肝に流入することで Kupffer 細胞がプライミングされ、再灌流時に再度腸管からのエンドトキシンなどに暴露されることで、いわゆる two hit phenomenon が惹起され、重篤な再灌流障害を被ると考えられるが、文献的にも死戦期をも考慮した心臓死肝移植の知見は未だない。本研究はあくまでも心臓死肝移植を臨床に応用するための基礎研究であり、いままでの心臓死腎移植の経験をふまえ、筋弛緩剤投与後に人工呼吸器を外すことで心臓死を誘導する、すなわち臨床により近い死線期を有する心臓死ドナーのモデルを採用している点が特徴である。

2.　心臓死肝移植における分子レベルでの障害発生機序の解明：
死戦期を伴う肝保存・再灌流傷害の機序を各種転写因子、サイトカイン、接着分子、アラキドン酸代謝等を解析することにより解明する。また、組織学的にも電顕レベル、免疫組織的手法で検討する。われわれのこれまでに得られた温阻血、冷保存、再灌流障害に関する知見と、本研究により得られた知見とを総合的に再評価・検討することで、臨床における心臓死肝移植を妨げる要因を明らかにし、その対策を探求する点に特徴がある。

3.　肝グラフトの viability を維持、改善する方法の開発の意義：
死戦期を伴う心臓死ドナーからの肝移植に関する以前の我々の検討では肝実質細胞におけるエネルギー産生能の再生には成功したが、逆に肝類洞の微小循環障害が惹起される結果となった（Endoh et al : Transplant.Proc., 1996)。しかし現在我々は心臓死ドナーの肝グラフトに対し虚血−再灌流障害発生においてその中心的役割を担う Kupffer 細胞を枯渇させ、さらに protease inhibitor を投与することで、虚血−再灌流後の肝類洞微小循環の維持することに成功している（Tsukamoto et al : Transplantation,1999)。これらの手法に加えて、分子レベルでの転写因子の変化を m RNA のアンチセンス投与などの遺伝子操作、IL1-ß, TNF∂阻害剤、protease inhibitor などの薬剤投与によりサイトカイン、アラキドン酸代謝を制御することでより長時間の冷保存が可能になると考えられる。このような研究に関しては未だ予防方法を確立したという報告はみられず本研究は独創的である。

本研究の意義

心臓死ドナーからの肝移植が臨床応用可能となれば肝グラフトの絶対数の増加につながり，本邦特有の死への概念を鑑みても本邦での移植医療を定着させていく上で重要な意味を持っている．また死戦期を被った脳死ドナーからの肝グラフトは高率にPGNを来すことも報告されている。本研究の成果はこのような不安定な脳死ドナーからのグラフト提供をも可能にすると期待され、本邦の移植医療を発展させる点で大きな意味を持つ。

本研究の位置づけ

1. 肝移植における肝実質細胞の障害発生機序の解明について：

心臓死ドナーからのグラフトと脳死もしくは生体部分肝移植におけるドナーからのグラフトとの明らかな相違はグラフトにかかる温阻血の有無である(Ohkohchi et al. Transplant. Proc, in press)。これまでの研究で我々は肝実質細胞は温阻血障害を受けやすく、冷保存-再灌流により類洞内皮細胞が障害を受けるとされることを明らかにした(Ohkohchi et al. Tohoku J exp Med, 1994)。当教室ではこれまでに、死線期を伴い心臓死をきたしたドナーの肝グラフトに対し人工心肺を用いて常温酸素化灌流を行うと、肝実質細胞におけるエネルギー産生能は著明に回復し、肝実質細胞の虚血性変化も回復することを明らかにした（Tsukamoto et al. Transplant. Proc, 1996)。その一方で常温酸素化灌流によると考えられる類洞内皮細胞障害が惹起されることを報告した（Endoh et al. Transplant Proc, 1996)。これらの結果より死線期を伴った心臓死ドナーからの肝グラフトであっても移植再灌流後の血流の維持、すなわち類洞構造という特殊な微細血管構造からなる肝微少循環を維持することができれば肝実質細胞は機能し心臓死ドナーからの肝移植も可能になると考えられる。

2. 肝移植における微少循環障害（微小血流障害）の障害発生機序について：

我々は、脳死肝移植における微少循環障害の発生機序に関する研究で、クッパー細胞の活性化に起因する活性酸素種と、その活性酸素種により誘導されるサイトカイン(TNF∂)を特定した（Shibuya et al. Hepatology, 1997)。また、好中球が類洞に集積するメカニズム、類洞内皮細胞障害の機序、ならびに肝実質細胞の脂質過酸化のメカニズムをすでに明らかにした（Terashima et al. Transplant. Proc, 1996、Shibuya et al. Hepatology, 1997, Ohkohchi et al. Transplantation, 1999)。また心臓死ドナーからの移植再灌流後の微少循環障害の発生機序として類洞腔の狭小化を確認しており、その原因としてはアラキドン酸カスケードを介した類洞の収縮と温阻血中の細胞内カルシウム増加に伴う肝実質細胞の膨化による二次的な類洞腔の狭小化が考えられ、この双方にフォスフォリパーゼＡ２が関与していることを報告した（Tsukamoto et al. Transplantation. 1999)。さらに死戦期において心機能の低下に伴う鬱血により中心静脈圧の高い状態が続くと、肝小葉の中心静脈域における類洞内皮細胞の剥離が認められる事を超微形態学的に確認している（Tsukamoto et al. Transplant. Proc, 1998)。

3. 肝移植における温阻血-再灌流時のサイトカインおよび核内転写因子の変化

心臓死ドナーからの移植再灌流後の血流障害、すなわち微少循環の障害発生機序に Kupffer 細胞から分泌される IL1-ß、thromboxane B2 が関与しており、肝組織中の NF-κB、AP-1 などの転写因子が発現することを明らかにした

(Oikawa et al. Transplant Int, 2000)。現在、死戦期を伴う心臓死ドナーからの肝移植を、死戦期（温阻血障害）、保存（冷阻血障害）、移植後の再灌流障害の 3 つの時期に分け、肝臓（主として Kupffer 細胞）においてサイトカインがどのように変化するか、ならびに核内転写因子の発現の変化を検討する準備を行っている段階である。加えて、rat を用いて温阻血障害時に発現する核内転写因子を抑制する薬剤、また障害に関与するサイトカインの分泌抑制作用を持つ薬剤の検討を行っている。

4. 肝 Kupffer 細胞機能の制御法について：

ラットを用いた実験でリポソーム封入クロドロネート投与により免疫染色で肝臓内の Kupffer 細胞が除去されることを確認した(Shibuya et al. Hepatology, 1997)。Kupffer 細胞を消去した場合温阻血肝において TNF∂の分泌抑制、 NFκB の発現低下を認め障害を軽減することを明らかにした（Oikawaet al. Transplant Int, 2000 ）。また、TNF∂の mRNA に対するアンチセンスを作成しリポソームに封入、Kupffer 細胞に貪食させ TNF∂の産生抑制効果も確認している。このような薬剤またはアンチセンスにより肝 Kupffer 細胞機能の制御を行った場合、投与後約一週間でペルオキシダーゼ陽性顆粒が出現し超微形態でも幼若なクッパー細胞を認め，投与後約二週間で完全に元に復することを確認している。アデノウィルスに比べ安全性の高いベクターである HVJ リポソームの副作用については、健常な rat に投与し血清生化学上異常のないことを，また肝エネルギー産生能についても影響を与えないことを確認している。目下保存中の HVJ リポソームによる肝グラフトに対して各種のアンチセンス導入の準備を行っている。

5. 死戦期を伴う心臓死肝移植について：

我々はブタを用いた心停止肝移植の実験で，リポソーム封入クロドロネート投与によりクッパー細胞を除去したうえで，プロテアーゼインヒビターを投与することにより，短時間の保存肝ではその生着率が 80%（無処置では生存率 0%）にまで劇的に改善することを報告した (Tsukamoto et al. Transplantation, 1999)。今後は、同実験系を用いて温阻血障害時に発現する核内転写因子をより効果的に抑制する薬剤、また障害に関与するサイトカインの分泌を強力に抑制する作用を持つアンチセンスや薬剤を投与し、その移植成績への効果を検討する予定である。

研究方法

平成１３年度

１、死戦期における肝温阻血障害発生機序の分子レベルでの解析（大河内、土井、小山田分担）

従来の心臓死モデルは KCl などの薬物による瞬間的心停止、大動脈の cross clamp による物理的肝血流停止が主なものであり、臨床的心停止における血圧低下や低酸素状態に暴露されるいわゆる死戦期を経ないモデルである（以下従来型モデルとする）。我々は、開胸することにより呼吸停止とし心臓停止を導く方法で心臓死モデルを作成した（以下死戦期モデルとする）。従来型では 140 分温疎血でも ラット肝移植の成功の報告もあり、一方死戦期モデルでは、全身ヘパリン化にもかかわらず、開胸後１時間程度の温疎血で門脈からの肝臓の wash out も不良となる。そこで、従来型モデルと死戦期モデルでの肝における温阻血障害発生機序の違いを IL1-ß, TNFα, IL-6 などの炎症性サイトカインを中心にその濃度の変化と関与する転写因子の発現動態、障害の初期に変化すると予想されるアラキドン酸カスケードの動きを 6keto PGF1∂, TBX, LTB4 などのエイコサノイドの変化から検討する。加えて炎症性リンパ球や活性化好中球の変化をインテグリン-ファミリーならびにセレクチン-ファミリーの接着分子の発現変化から検討する。

２、死戦期のを伴った心臓死ドナー肝グラフトにおける冷保存-再灌流障害発生機序の分子レベルでの解析（肝 Kupffer 細胞の果たす役割を中心に）（大河内、藤盛、小山田分担）

予備実験において、全身ヘパリン化を行っても、死戦期モデルにおいては、開胸後 45 分から 1 時間経過すると、門脈血の wash out が不良になるが、しかし、リポゾームクロドロネートにて Kupper 細胞をあらかじめ消去した場合では約 1 時間後でも wash out される。また、これまでの検討では、温阻血再潅流モデル、エンドトキシン注入モデルにおいて約 1 時間という早い段階で NF-kB, AP-1 といった転写因子の活性化、 E-selectin mRNA の発現を確認しており、温阻血１時間の間に微小循環障害につながるこれらの変化が肝内で既に起きている事が強く示唆される。そこで、無処置群と Kupffer 細胞消去群において、実験 1 で作成したラット死戦期モデルの肝グラフトを用いて保存、移植を行い i) NF-kappaB, AP-1 などの転写因子の活性変化、MAP キナーゼの活性変化、c jun キナーゼ（ JNK ）や MAP キナーゼファミリーの上流に位置する MEKK-1 やホモログの NIK などの核内転写因子およびキナーゼ群の活

性変化、 ii) NF-kappaB により誘導されるサイトカイン（ IL-1, IL-2, IL-6, GM-CSF, TNF など）とそれに関わる NFIL-6 などの転写因子の発現動態、接着分子（ E-selectin, P-selectin, LFA1, ICAM-1, VCAM-1 など)、NOS 、Fas ligand などの発現変化、iii) 免疫応答や炎症反応の際活性化されるアラキドン酸代謝の key enzyme であるホスホリパーゼ A2 活性変化およびその代謝産物であるエイコサノイドの変化を検討し、総合的に温阻血、冷保存、再潅流障害の発生機序を分子レベルで解明する。

これらの測定において、これまでの検討では転写因子の測定にアイソトープを用いていたが、今後は多数の検体を測定する必要があるため non-isotopic に測定が可能な non-R I 発光核酸および蛋白検出システムを備品として請求した。

H１４年度

３、死戦期を伴う心停止ドナー肝の冷保存・再潅流障害に対するアンチセンス、薬剤の投与効果の検討（大河内、土井、小山田分担）

死戦期を伴う心停止ドナー肝におこる障害の発生機序の鍵を握る Kupffer 細胞の消去治療をあらかじめドナーに行うことは臨床では倫理的に許されない。死戦期を伴う心臓死ドナーからの肝移植を臨床応用するためには、Kupffer 細胞の活性を強く抑制、サイトカインの分泌抑制、核内転写因子の発現を抑制、アラキドン酸カスケードの活性化の抑制の開発が必要不可欠となる。実験１の死戦期を経た心臓死モデルの肝グラフトを移植し、実験２から得られた結果に基づき、IL-1ß, IL-2, IL-6, TNF α などの mRNA のアンチセンス、サイトカイン分泌阻害剤および抗体、フォスフォリパーゼ A2 阻害剤、NF κ B 活性抑制を目的とした各種 protease inhibitor、アラキドン酸の代謝産物である PGI2 および E1 の投与効果, その投与時期について生着率、肝機能に加えて各種転写因子の発現、サイトカインの分泌、接着分子の発現、ならびにアラキドン酸代謝等を解析することにより比較検討する。また、電顕を用いて肝類洞を中心に微細構造についても検討する。その際、単独投与の効果のみではなく併用効果についても検討する。

４、臨床応用を前提とした大動物（豚）を用いた死戦期を伴う心臓死ドナー肝の障害に対する薬剤・遺伝子治療の効果の検討（大河内、藤盛、川岸、土井分担）

実験 3 の結果に基づき、臨床応用を考え豚の死戦期を伴う心臓死ドナーからの肝移植モデルを用いて i) 遺伝子操作（mRNA に対する HVJ リポゾームを用いたアンチセンス導入）によるサイトカイン分泌抑制および各種転写因子の制

御、ii）薬剤による Kupffer 細胞機能（サイトカイン分泌、活性酸素放出）の抑制効果、iii）protease-inhibitor やプロスタグランジンの投与効果による肝温阻血障害抑制効果を検討する。加えて、これらの操作による免疫応答や炎症反応の抑制効果についても検討し臨床応用が可能な安全かつ有効な方法の確立を目標とする。

研究結果

課題１及び２：死戦期のを伴った心臓死ドナー肝グラフトにおける冷保存-再灌流障害発生機序の分子レベルでの解析（肝 Kupffer 細胞の果たす役割を中心に）

【方法】実験１）好中球の役割について：好中球添加液をもちいたラット肝灌流実験において脂質過酸化障害のメカニズムを検討した。実験２）クッパー細胞の役割について：クッパー細胞を刺激または抑制したラット肝灌流実験でにおいて、冷保存再灌流障害を活性酸素産生、脂質過酸化、好中球集積に着目し検討した。実験３）心停止肝移植における温阻血障害部位の検討と改善について：ブタ心停止肝移植モデルで、温阻血冷保存による肝細胞障害をエネルギー産生能と電顕で検討し、さらに人工心肺装置を用いたプレコンディショニングを施行した。実験４）心停止肝移植における類洞内皮障害の予防法の開発：クッパー細胞消去モデルを用いブタ心停止肝移植実験を行った。実験５）温阻血冷保存再灌流障害機序について：ラット心停止モデルを用いた灌流実験で各種転写因子、サイトカイン、エイコサノイドの挙動を検討した。

【結果】実験１）好中球を添加することで脂質過酸化が増強した。実験２）活性酸素産生、脂質過酸化ともにクッパー細胞抑制系で減弱し、クッパー細胞刺激系で増強した。またクッパー細胞抑制系では好中球の集積が抑えられた。実験３）人工心肺装置による再酸素化で肝細胞のエネルギー産生能は回復したが、冷保存することで著明な類洞内皮細胞障害が惹起された。実験４）クッパー細胞消去により類洞内皮細胞障害は改善したが、類洞狭小化による微小循環障害により生存しなかった。しかし、クッパー細胞消去にプロテーゼインヒビター含有リンス液を併用することで生存した。実験５）心停止モデルにおいてのみ、再灌流時、転写因子 NF-kB 活性の増強をみた。また心停止モデルで IL-1β 産生が有意に高く、エイコサノイド産生もトロンボキサン優位の産生パターンを示した。

【まとめ】肝移植における再灌流障害ではクッパー細胞が強く関与し、特に温阻血が加わる心停止肝移植ではその制御が重要な課題になる。

課題３：死戦期を伴う心停止ドナー肝の冷保存・再灌流障害に対する薬剤の投与効果の検討

【方法】雄性 Wistar rat を使用し、５群に分け肝摘出再灌流実験を施行した。
1)Heart beating 群(HB 群)：心拍動下に肝摘出。6 時間冷保存後再灌流。
2)Non-heart-beating 群(NHB 群)：呼吸停止から心停止を誘導し、心停止下に

肝摘出。6 時間冷保存後再灌流。3)nafamostat mesilate 投与群(NM 群)：nafamostat mesilate 投与後,NHB 群と同様に肝摘出。冷保存後再灌流。4)FR167653 投与群(FR 群)： FR167653 投与後,NHB 群と同様に肝摘出。冷保存後再灌流。4)FR167653+ nafamostat mesilate 投与群(FR+NM 群)：nafamostat mesilate 及び FR167653 投与後,NHB 群と同様に肝摘出。冷保存後再灌流。5 群とも灌流時間は 1 時間としその後にサンプルを採取した。検査項目は、胆汁産生量、灌流液量、生化学検査、IL-1β,TNF-α, thromboxaneB2(TXB2), 6-ketoPGF1α, leukotrieneB4, Cox-2, NF-κB, AP-1, 病理組織(電顕、光顕)である。

【結果】HB 群と比し、NHB 群で IL-1β,TNF-α,NF-κB, AP-1, TXB2, leukotrieneB4, Cox-2 が有意に高く、FR+NM 群では NHB 群に比し有意に低下した。病理所見でも FR, FR+NM 群で肝類洞の微小循環障害が改善された。以上の結果から、FR167653 と nafamostat mesilate は、温阻血再灌流障害時において、炎症性サイトカイン及びアラキドン酸カスケードのメディエーターを著明に抑制することが確認された。また、この 2 剤併用療法は、屍体肝グラフトの肝類洞における微小循環保持に極めて有用であることが示唆された。

【結語】心停止ドナーからの肝移植では、死戦期（温阻血期）における IL-1β・TNF-α の抑制およびアラキドン酸カスケードの抑制が重要であると考えられた。FR167653 と nafamostat mesilate の 2 剤併用療法は、屍体肝グラフトのコンディショニング法として効果があると考えられた。

＊アンチセンスの効果は検討できなかった。

課題４：臨床応用を前提とした大動物（豚）を用いた死戦期を伴う心臓死ドナー肝の障害に対する薬剤の効果の検討

【方法】ブタを用い以下の５つの群に分け実験を行った.（１）Control 群：以下に述べる薬剤を用いず心停止肝移植を行った群.（２）FR 群：再灌流障害の軽減を目的として、TNF-α および IL-1β の阻害剤である FR167653（FR）の投与を行い心停止肝移植を行った群.（３）PG 群：温阻血障害発生の予防を目的として、プロスタグランジン E1（PG）の投与を行い心停止肝移植を行った群.（４）FRPG 群：FR、PG の双方を投与し心停止肝移植を行った群.（５）HBD 群：positive control として心拍動下肝移植を行った群。

【結果】PG 投与によって温阻血障害が抑制され、FR 投与によって再灌流障害が軽減されたが、これらの薬剤は単独の投与では生存率の向上は得られなかった. PG と FR の双方を投与することによって心拍動下肝移植と同等の生存率が得られた。

【結論】心停止ドナーからの肝移植では心拍動下の移植と異なり、温阻血障害の存在とこれに引き続くより高度の再灌流障害が問題となる．本研究ではこれらが臨床応用可能な薬剤によって制御可能であることを示唆し、その結果、心停止肝移植を安全に行うことが可能であると示唆された。
＊遺伝子治療については以上の研究結果を詳細に検討した上で今後検討を行う予定である。

課題１及び２：死戦期のを伴った心臓死ドナー肝グラフトにおける冷保存-再灌流障害発生機序の分子レベルでの解析（肝 Kupffer 細胞の果たす役割を中心に）

Transpl Int (2002) 15: 397–405
DOI 10.1007/s00147-002-0435-8

ORIGINAL ARTICLE

Kosei Oikawa
Nobuhiro Ohkohchi
Masahide Sato
Atsuschi Masamume
Susumu Satomi

Kupffer cells play an important role in the cytokine production and activation of nuclear factors of liver grafts from non-heart-beating donors

Received: 3 September 2001
Revised: 5 April 2002
Accepted: 24 April 2002
Published online: 10 August 2002
© Springer-Verlag 2002

K. Oikawa · M. Sato · S. Satomi
Division of Advanced Surgical Science, Tohoku University, Graduate School of Medicine, Seiryo-machi 1-1 Aoba-ku, Sendai, Japan

N. Ohkohchi (✉)
Department of Gastrointestinal Surgery, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Japan
E-mail: nokochi3@md.tsukuba.ac.jp

A. Masamume
Division of Gastrontestinal Disease, Tohoku University, Graduate School of Medicine, Seiryo-machi 1-1 Aoba-ku, Sendai, Japan

Abstract In the non-heart-beating donor (NHBD) deterioration of microcirculation of the liver graft is strongly associated with secretion of cytokines and eicosanoids. In this study we investigated the excretion of cytokines, eicosanoids, and DNA binding activity of transcription factors in the grafts from NHBD and evaluated the effects of the elimination of Kupffer cells on them. The purpose of this study was to clarify the impact of Kupffer cells on transcription factor activity and the that of cytokine and eicosanoid production on reperfusion injury of liver grafts from NHBD. Wistar rats were allocated to four groups: (a) control group: livers were extracted under heart-beating conditions and perfused without cold storage, (b) heart beating (HB) group: livers extracted under heart-beating conditions were perfused after 6 h of cold storage, (c) non-heart-beating (NHB) group: livers extracted after cardiac arrest were perfused after cold storage, (d) Kupffer cell eliminated (KE) group: liposome-encapsulated dichloromethylene diphosphonate was intravenously administered to eliminate Kupffer cells before extraction, and the liver was perfused after cold storage. Cytokines and eicosanoids in perfusate were measured. DNA binding activity of nuclear factor κB, activating protein 1, and nuclear factor–interleukin 6 of tissue were investigated. Concentrations of interleukin 1β and thromboxane B_2 in the perfusate were significantly higher in NHB group, but they were completely suppressed in the KE group. A rise in binding activity of nuclear factor κB and activating protein 1 was not observed during cold storage in any groups, but these activities did increase remarkably after reperfusion. Significant buildup of those activities were recognized in the NHB group, and this phenomenon was inhibited in the KE group. The histological structures of the sinusoid in the KE group were well maintained, as with those of the control group. These results indicate that cytokines, eicosanoids, and the DNA binding activity of the transcripton factor are strongly associated with reperfusion injury, and Kupffer cells play an important role in this mechanism in grafts from NHBDs.

Keywords Non-heart-beating donor · Liver transplantation · Cytokine · Eicosanoid · Nuclear transcription factor · Kupffer cell

Introduction

Liver transplantation, which is established as a therapy for end-stage hepatic disease, has become a common surgical treatment all over the world. Due to technical progress and the development of immunosuppressive agents the outcome of liver transplantations has improved markedly, and the indications for transplantation have been extended. However, donor numbers do not increase in most countries, and graft shortage has become a major problem, especially in Europe and North America. Consequently the utilization of grafts from so-called marginal donors, which would previously have been classified as unsuitable for transplantation, are now being considered [20], and a special program has been devoted to the use of liver grafts from donors with cardiac arrest [8]. According to a prospective study, the number of donors would increase by 20–25% if donation of liver grafts from marginal donors were to be introduced [22]. At present, however, transplantation of liver grafts from cardiac arrest donors is rarely carried out because of the high incidence of primary graft nonfunction (PGN).

Ischemic reperfusion injury of the liver graft subjected to cold preservation is considered one of the main factors of PGN [10]. It has been reported that PGN is strongly associated with the involvement of the impaired sinusoidal endothelial and activated Kupffer cells [6, 7, 30, 31, 36]. We have already reported that active oxygen production, lipid peroxidation, and activation of both neutrophils and Kupffer cells are strongly associated with the mechanism of PGN [16, 23, 24, 25, 30, 31, 34, 35, 37]. Conditions such as an agonal state of hypoxemia, hypotension, abnormal electrolyte levels, and acidosis seem to induce serious biological reactions by the transplanted liver graft. The reactions caused by hypoxia and acidosis in NHBD are probably more complicated than those induced by simple cold storage and reperfusion injury [41]. Especially the increase in inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin (IL) 1β, and IL-6 as well as the increment of DNA binding activity of transcription factors, such as nuclear factor κB (NF-κB) and activating protein 1 (AP-1) are strongly associated with the mechanism of graft injury [5, 26, 33].

The purpose of the present study was to investigate the mechanism of ischemic reperfusion injury in liver grafts in a cardiac arrest (NHBD) model focusing on inflammatory cytokines, eicosanoids, and the DNA binding activity of transcription factors. We furthermore wanted to clarify the role of Kupffer cells in ischemic reperfusion injury.

Materials and methods

Experimental design

Male Wistar rats (Charles River, Tokyo, Japan) weighing 250–300 g were used. Under anesthesia with ether the livers was extracted and preserved with and without cold preservation. Livers were divided into the following four groups according to the surgical procedure and subsequent treatment [14]. (a) In the control group, the livers were extracted under heart-beating conditions and perfused without cold storage. (b) In the heart-beating (HB) group, the livers were extracted under heart-beating conditions and after 6 h of cold storage were perfused with University of Wisconsin (UW) solution. (c) In the non-heart-beating (NHB) group, livers were extracted after cardiac arrest and after 6 h of cold preservation were perfused. (d) In the Kupffer cell eliminated (KE) group, liposome-encapsulated dichloromethylene diphosphonate (5 ml/kg) was intravenously administered to eliminate Kupffer cells 42 h before hepatectomy. In this group, the livers were extracted after cardiac arrest and perfused after 6 h of cold storage. Each group consisted of 6 rats.

Experimental procedure

After heparinization, 50 mg/kg of Nembutal was intraperitoneally administered to induce general anesthesia. The abdomen was opened with a midline incision. After introduction of a silicone tube into the common bile duct, the sheath of a 12 gauge needle was inserted into the portal vein. The inferior vena cava was incised, and the portal blood was washed out with 10 ml of physiological saline at 4°C and UW solution under a pressure of approximately 10 cmH_2O. Then, the liver was preserved in UW solution at 4°C.

Induction of cardiac arrest

The diaphragm was incised to induce respiratory stop and cardiac arrest. The rat was left at room temperature for 30 min. Cardiac arrest was induced 10.0 ± 1.2 (mean ±standard) min after the thoracotomy. Afterwards, the liver was washed out via the portal vein and extracted.

Elimination of Kupffer cells

Dichloromethylene diphosphonate was prepared in accordance with the method described by Van Rooijen [38]. In 20 ml methanol chloroform (1:1) 75 mg phosphatidylcholine (Sigma, St. Louis, Mo., USA) and 11 mg cholesterol (Sigma) were dissolved, and the mixture was dried in an evaporator under low pressure at 37°C. This procedure was repeated twice. A thin phosphatidylcholine and cholesterol membrane was formed in a flask. Of dichloromethylene diphosphonate (Boehringer-Mannheim, Laval, Quebec, Canada) 1.89 g was dissolved in phosphate-buffered solution (PBS), pH 7.4 at 23°C. The whitish thin membrane was dissolved with the mixed solution by shaking the flask for 1 h in a water bath at 68°C. Nitrogen gas was placed on the surface, and the solution was left at room temperature for 2 h. Then the solution was passed through a 0.6-μm filter. To remove dichloromethylene diphosphonate which was not encapsulated in the liposome by this procedure the solution was diluted with 100 ml PBS and centrifuged at 5500 rpm for 30 min. The supernatant was diluted with 20 ml PBS and centrifuged at 100,000 *g* for 30 min. After the supernatant was discarded, the pellet was suspended in 4 ml PBS. This final solution was administered intravenously to rats at a dose of 5 ml/kg body weight 42 h before hepatectomy. The effect of dichloromethylene diphosphonate on Kupffer cells has been reported previously, and

the validity was confirmed by immunohistological stain using ED2 antibody and colloid carbon phagocytosis [30].

Perfusion of the liver in vitro

The schema of the reperfusion system of the liver in vitro is shown in Fig. 1. The perfusion circuit was filled with Krebs-Henseleite bicarbonate buffer which was filtrated through a 0.2 µm filter. An oxygen and carbon dioxide gas mixture (95% O_2 + 5% CO_2) was bubbled into the buffer. The buffer was perfused into the portal vein of the liver at a pressure of 10 cmH_2O for 60 min. Liver specimens were taken under heart beating conditions, after cardiac arrest, during cold preservation (0, 6, 12, 24 h), and 60 min after perfusion. The specimens were freeze-clamped in liquid nitrogen. The frozen tissue sample was stored at −80°C until measurement of DNA binding activity of transcription factor.

Eicosanoids

Thromboxane (TX) B_2, 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) and leukotriene B_4 that were released into the perfusate from the liver were assessed by measuring eicosanoids concentration in the perfusate 60 min after reperfusion using commercially available, enzyme-linked immunosorbent assay kits (Cayman Chemical, Ann Arbor, Mich., USA).

Cytokines

Inflammatory cytokines, i.e., TNF, IL-1, and IL-6 that were released into the perfusate from the liver, were assessed by measuring cytokine concentration in the perfusate 60 min after reperfusion, using commercially available enzyme-linked immunosorbent assay kits (Biosource International, Camarillo, Calif., USA).

Extraction of nuclei from liver tissue

The nuclei were extracted according to the method described by Dignam et al. [11]. The following procedure was conducted at 4°C. The tissue was dissolved in 5 ml of a solution, consisting of 0.6% IGEPAL CA-630, i.e., a nonionic detergent, 150 mM NaCl, 10 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES) pH 7.9, 1 mM ethylene diaminetetraacetate (EDTA), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). A pestle (Dounce tissue grinder, Wheaton, Millville, N.J., USA) was used to homogenize the sample approximately gently ten times. To remove uncrushed tissues the homogenate was centrifuged at 2000 rpm for 1 min. The supernatant was centrifuged at 5000 rpm for 5 min. The precipitated nuclei were introduced into a solution which consisted of 25% glycerol, 20 mM HEPES at pH 7.9, 420 mM NaCl, 1.2 M $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 2 mM benzamidine, 5 µg/ml pepstatin, 5 µg/ml leupeptin, and 5 µg/ml aprotinin, and the mixture was left on the ice for 20 min. The solution containing nuclei was centrifuged at 6,000 rpm, and the supernatant was collected. Then the sample was stored at −80°C. Nucleoprotein was quantified using a commercially available BCA protein assay kit (Pierce Chemical, Rockford, Ill., USA).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was carried out to investigate the DNA binding activity of NF-κB, AP-1, and NF IL-6 (C/EBP) of the liver specimens. Double-stranded oligonucleotides were prepared as follows: NF-κB: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, AP-1: 5′-CGC TTG ATG AGT CAG CCG GAA-3′ (Promega., Madison, Wis., USA), C/EBP: 5′-TGC AGA TTG CGC AAT CTG CA-3′ (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), and T4 polynucleotide kinase (Promega). [^{32}P]ATP was incubated with these oligonucleotides at 37°C for 30 min for labeling and used as a probe. Then the sample was purified through Quick Spin G-25 column (Boehringer-Mannheim, Piscataway, N.J., USA). The liquid nuclear extract of 15 µg protein and the probe were incubated with the binding reaction buffer, which consisted of 10 mM Tris-Cl at pH 7.5, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 4% glycerol, and 0.1 mg/ml polydeoxyinosinic deoxycytidylic acid at room temperature for 20 min. For the supershift assay, before the addition of the probe, NF-κB was incubated with anti-p50, p65, and c-Rel antibodies (2 g/sample: Santa Cruz Biotechnology) at 4°C for 1 h. For the competition assay, before the addition of the probe, unlabeled oligonucleotide and mutant oligonucleotide were incubated at room temperature for 10 min. The sample was electrophoresed in 4% polyacrylamide gel with Tris-borate-EDTA buffer under 150 V for 2 h. After drying the gel was exposed to the film at −80°C.

Histological findings

Sixty minutes after perfusion the specimens of the liver were fixed in 10% neutral formalin, and hematoxylin and eosin staining was carried out.

Statistics

All values and figures are the mean ± standard deviation (SD). Statistical analysis was carried out using Student's *t* test. Differences at the level of $P < 0.05$ were considered statistically significant.

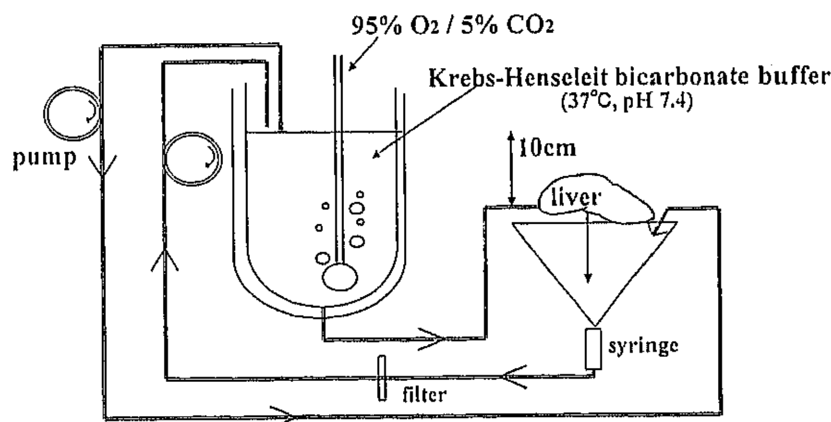


Fig. 1. Scheme of the perfusion system of the liver. The liver was perfused for 60 min under a pressure of 10 cmH_2O with 100 ml Krebs-Henseleit bicarbonate buffer (37°C, pH 7.4) saturated with 95% O_2 and 5% CO_2 mixture in a blood-free recirculating system

Results

Eicosanoids in perfusate

The concentration of TXB_2 was significantly higher in the HB and NHB groups than in the other two groups; in the KE group it was completely suppressed, as was that of IL-1 (Table 1). The concentration of 6-keto $PGF_{1\alpha}$ was significantly higher in the HB group than in the other groups; in the NHB group it was reduced to the level in the control group. The ratio of TXB_2 to 6-keto $PGF_{1\alpha}$ was significantly higher in the NHB group than in the other groups; in the KE group it was completely suppressed (Table 1). Leukotriene B_4 was not detected in any of the groups (data were not shown).

Cytokines in perfusate

The concentration of IL-1β was significantly higher in the NHB group than in the other groups; in the KE group the production of IL-1β suppressed to the same level in the control group (Table 1). The values in the HB and NHB groups were slightly elevated, but there was no statistically significant difference between group. In the KE group, however, the production of TNFα was suppressed to the same level as in the control group (Table 1). IL-6 was not detected in any of the groups (data were not shown).

Transcriptions factor

No NF-κB activity was observed, in spite of cold preservation in each group. After 60 min of reperfusion activity in the NHB group was clearly higher than in the other groups. Activity in the KE group was lower than in the NHB group. In the competition and supershift assays of the nuclear extract from the NHB group, the band was abolished by 100-fold excess of unlabeled wild-type oligonucleotide (lane wt). In contrast, the mutated oligonucleotide had no effect on the band in the NHB group (lane mt). Supershift assay with antibodies against p50, p65, and c-Rel showed the identity of different NF-κB members. In the NHB group the p50/p50 homodimer was dominant (Fig. 2).

No AP-1 activity was observed in spite of cold preservation. Activity after 60 min of reperfusion was markedly higher in the NHB group than in the other groups. Activity in the KE group was lower than in the NHB group. In competition assays the band of NHB was abolished by 100-fold excess of unlabeled wild- type oligonucleotide (lane wt). In contrast, the mutated oligonucleotide had no effect on the band in the NHB group (lane mt; Fig. 3).

DNA binding activity of NF-IL6 was observed during cold preservation, and after 60 min of reperfusion the binding activity remained unchanged in all three groups. In the competition assay the band of NHB was abolished by 100-fold excess of unlabeled wild-type oligonucleotide (lane wt). In contrast, the mutated oligonucleotide had no effect on the band in the NHB group (lane mt; Fig. 4).

Histological findings

In the HB group the structure of the liver was well-maintained, although a slight disorder was recognized in the perisinusoidal area. In the NHB group the sinusoidal structure was remarkably deteriorated, and a hydropic change in parenchymal hepatocytes was observed. The histological structure was well maintained in both the control and KE groups (Fig. 5).

Discussion

The use of liver grafts from NHBD is still a matter of controversy in clinical transplantation. In some

Table 1. Cytokines and eicosanoids in perfusate 60 min after reperfusion (*HB* heart beating group: the liver, extracted under heart beating conditions, was perfused after 6 h of cold storage with solution, *NHB* non-heart-beating group: the liver extracted after cardiac arrest was perfused after 6 h of cold preservation, *KE* Kupffer cell eliminated group: liposome-encapsulated dichloromethylene diphosphonate was administered intravenously to eliminate Kupffer cells 24 h before extraction of the liver, and perfused after 6 h of cold storage)

Group	Control	HB	NHB	KE
Eicosanoid				
6-keto $PGF_{1\alpha}$ (pg/ml)	178+72	346+119*	150+30	120+50
TXB_2 (pg/ml)	81+26	2017+525**	2703+1380**	17+18
TXB_2/6-keto $PGF_{1\alpha}$ ratio	0.4±0.1	4.3±2.3	18.6±9.6*	0.1±0.1
Cytokine				
IL-1β (pg/ml)	34.2+13.0	32.8+12.2	61.4+14.7*	40.6+18.5
TNFα (pg/ml)	0.3+0.1	4.5+6.3	3.2+5.5	0.4+0.6

* $P<0.05$ versus all other groups,
** $P<0.05$ versus KE group

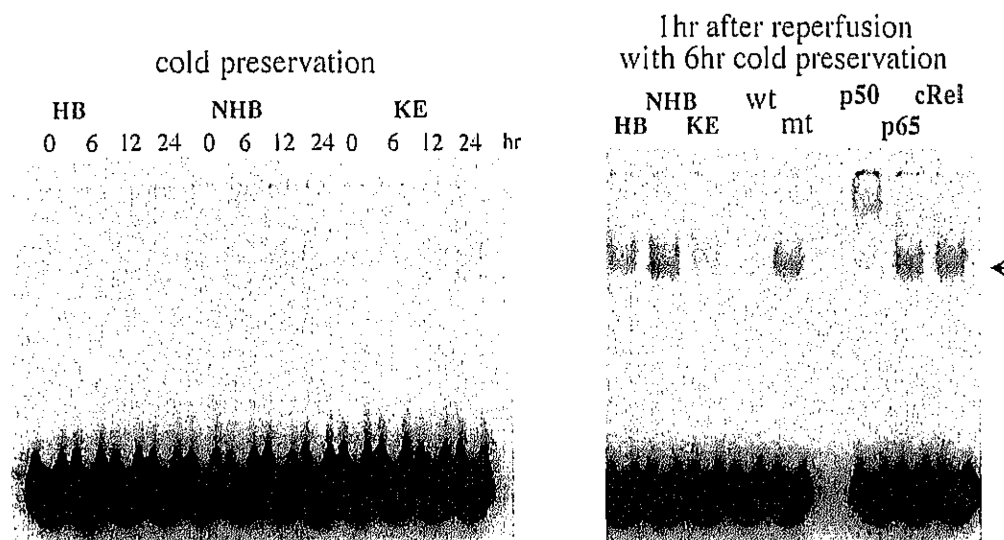


Fig. 2. Electrophoretic mobility assay of NF-κB activation. No activity was observed in spite of cold preservation in each group. Activity after 60 min of reperfusion was markedly higher in the NHB group and lower in the KE group than in other groups. In the competition and supershift assays of the nuclear extract from the NHB group the band was abolished by 100-fold excess of unlabeled wild-type oligonucleotide (*lane wt*). In contrast, the mutated oligonucleotide had no effect on the band in the NHB group (*lane mt*). Supershift assay with antibodies against p50, p65, and c-Rel showed the identity of different NF-κB members. In the NHB group the p50/p50 homodimer was dominant. *HB* Heart-beating donor with 6 h cold preservation; *NHB* non-heart-beating donor with 6 h of cold preservation; *KE* non-heart-beating donor with elimination of Kupffer cells and 6 h of cold preservation; *wt* wild type; *mt* mutated type

countries liver transplantation from NHBD is carried out in urgent cases as a trial, but transplantation results are still unsatisfactory. Such livers are usually not used in urgent cases, but in stable patients who are able to withstand the greater reperfusion injury of the liver from NHBD, according to the recent North American report. Injury of the liver graft from NHBD is associated with various cytokines released from activated Kupffer cells [26]. We have previously reported that Kupffer cells play an important role in reperfusion injury of the liver graft from NHBD after cold preservation [37]. In this study we found that IL-1β and TXB_2 concentrations are significantly higher in liver grafts from NHBD during reperfusion after cold preservation in NHBD liver graft than in with prostacyclin. We also observed that transcription factors, i.e., NF-κB and AP-1, were not activated during cold preservation, but were after reperfusion. The activation was enhanced especially in agonal NHBD. These phenomena were not induced by the elimination of Kupffer cells, and as a result reperfusion injury of sinusoidal microcirculation was effectively prevented. As this study suggests, the key to successful transplantation from NHBD is likely control of Kupffer cells function.

There are several models of NHBD, i.e., induction with respirator off, thoracotomy, and administration of KCl. Zhang et al. [41] reported that in the NHBD model induced by administration of KCl, arterial oxygen content and ability of ATP synthesis in mitochondria 30 min after induction were significantly higher than those in the NHBD model induced by the thoracotomy. Therefore in this study we chose the NHBD model induced by thoracotomy in which liver graft suffered from agonal conditions such as hypoxia.

According to previous studies on reperfusion injury of the liver after cold preservation, the parenchymal hepatocytes deteriorated as a result of the damage of sinusoidal endothelial cells [20]. The mechanism of this phenomenon has been explained as follows. ATP depletion [12, 19] and the increase in intracellular calcium activate various enzymes and affect the cellular structure and the permeability of cell membrane [13, 15, 21]. In addition, metabolites in the arachidonic acid cascade are also associated with the deterioration in sinusoidal endothelial cells and coagulopathy of sinusoid. TXA_2, which derives from the activation of phospholipase A_2, has strong effects of platelets aggregation and vasoconstriction [28] and induces the accumulation of neutrophils in sinusoid [18, 39, 40]. Moreover, TXA_2 impairs the microcirculation of the liver directly, and treatment by inhibition of TXA_2 production improves the microcirculation after reperfusion in the ischemic liver [32]. Post et al. [27] reported that the level of prostanoids in hepatic veins increased 100–500 times after reperfusion. TXA_2 and PGI_2 are unstable in the blood and metabolized to TXB_2 and 6-keto PGF_1, respectively. This study clearly indicated the increase in prostacyclin, which induced vasodilation in microcirculatory disturbance of the liver after reperfusion following cold preservation. We also showed that TX is significantly higher in the liver graft from NHBD than prostacyclin. Therefore, in view of the

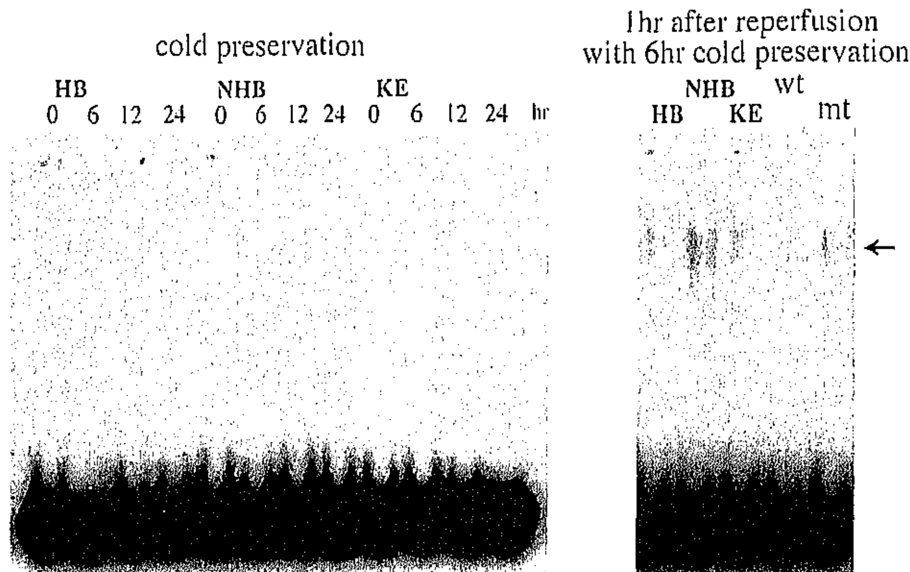


Fig. 3. Electrophoretic mobility assays for AP-1 activation. No activity was observed in spite of cold preservation in each group. Activity after 60 min of reperfusion was markedly higher in the NHB group and lower in the KE group than in other groups. In competition assays the band of NHB was abolished by 100-fold excess of unlabeled wild-type oligonucleotide (*lane wt*). In contrast, the mutated oligonucleotide had no effect on the band in the NHB group (*lane mt*). *HB* Heart-beating donor with 6 h cold preservation; *NHB* non-heart-beating donor with 6 h cold preservation; *KE* non-heart-beating donor with elimination of Kupffer cells and 6 h cold preservation; *wt* wild type; *mt* mutated type

arachidonic acid metabolism, the agonal condition of the NHBD donor has severe adverse effects on the sinusoidal microcirculation in the liver, as opposed to the heart beating donor. In addition, we demonstrated that Kupffer cells play important roles in activation of the arachidonic acid metabolism in the graft from NHBD.

Arachidonic acid, which is a component of the cell membrane, is a substrate metabolized through the arachidonic acid cascade. The rate-limiting enzymes involved in this cascade are divided into cyclo-oxygenases (COX) and lipoxygenases. COX metabolizes arachidonic acid into PGG_2. PGG_2 is metabolized from PGH_2, PGE_2, and PGD_2 to PGF_2. In addition to this process, there are two other metabolic processes, and the final metabolites are PGI_2 and TXA_2. Caughey et al. [9] reported that the productions of IL-1β and TNFα are inhibited by TXA_2 in human monocytes, and suggested activation of the arachidonic acid cascade by signal transduction. Two COX isozymes have been identified, i.e., COX-1 and COX-2. COX-1 is a constitutive protein in almost all the cells. On the other hand, COX-2 is an inducible protein. NF-κB, AP-1, and NF IL-6 exist in

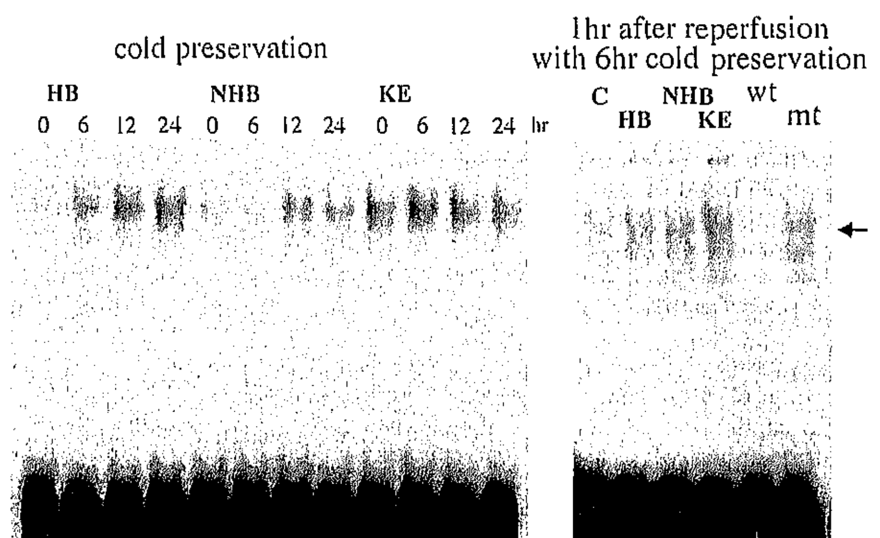


Fig. 4. Electrophoretic mobility assays for NF-IL6 activation. DNA-binding activity was observed during cold preservation and after 60 min of reperfusion the binding activity remained unchanged in the three groups. In the competition assay the band of NHB was abolished by 100-fold excess of unlabeled wild-type oligonucleotide (*lane wt*). In contrast, the mutated oligonucleotide had no effect on the band in the NHB group (*lane mt*). *HB* Heart-beating donor with 6 h cold preservation; *NHB* non-heart-beating donor with 6 h cold preservation; *KE* non-heart-beating donor with elimination of Kupffer cells and 6 h cold preservation; *wt* wild type; *mt* mutated type

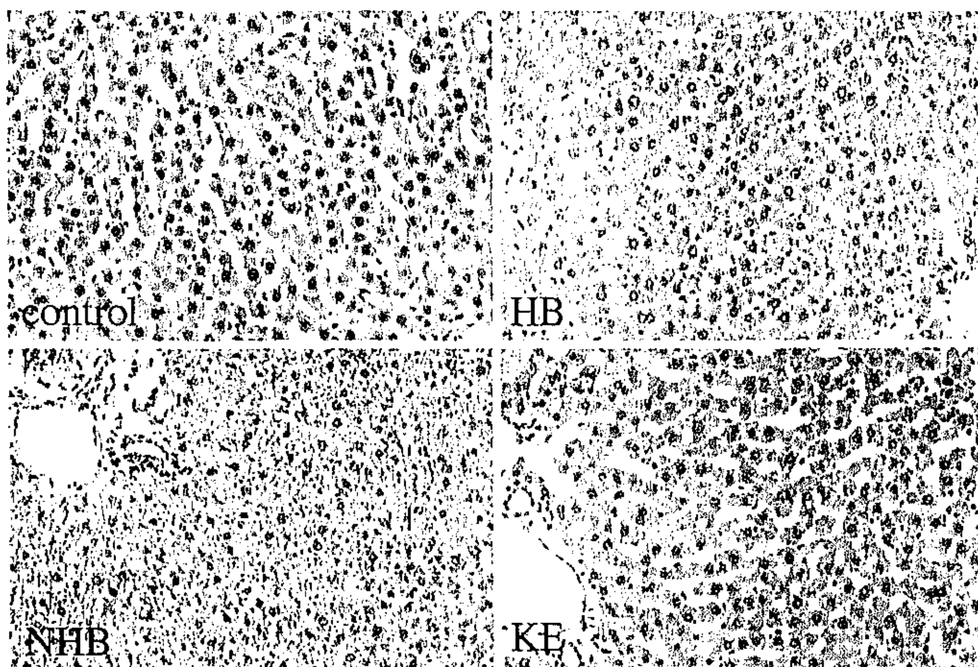


Fig. 5. Morphological findings in the livers 60 min after reperfusion. In the NHB group sinusoidal spaces became narrow, and hydropic changes were observed in hepatocytes. In the KE group sinusoidal structures and hepatocytes were well preserved, as in the control group. *Control* Heart-beating donor without preservation; *HB* heart-beating donor with 6 h cold preservation; *NHB* non-heart-beating donor with 6 h cold preservation; *KE* non-heart-beating donor with elimination of Kupffer cells and 6 h cold preservation. Hematoxylin and eosin stain, ×100

the promoter region of COX-2, and COX-2 is induced by IL-1 and NF-κB [1]. Although we did not examine COX-2 expression, the results of our study, i.e., increment of eicosanoid production and inflammatory cytokines and enhancement of transcription factor activity, suggest that cold preservation/reperfusion injury in the graft from NHBD is associated with COX-2 induction in the liver graft.

Many studies have been carried out recently on the mechanism of biological reactions, including acute-phase inflammation. Attention has been drawn especially to transcription factors, which bind to the promoter of gene expression and induce transcription activity. NF-κB was the first transcription factor identified as a nucleoprotein that binds to the enhancer of immunoglobulin k chain genes and was regarded as a B cell specific transcription factor [29]. Subsequent studies demonstrated that NF-κB is a transcription factor which controls the activities of various cytokines, i.e., IL-1, IL-2, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, TNFα, COX-2, chemokine, adhesion molecules such as E-selectin, intercellular adhesion molecule 1, vascular cell adhesion molecule, inducible nitric oxide synthase, Fas ligand, IL-2 receptor, and gene expression, as well as human immunodeficiency virus and cytomegalovirus self-replication. NF-κB plays an important role in preventing apoptosis induced by TNFα [2]. There has been a report that massive liver-cell death was induced in NF-κB knockout mice, and this indicates the necessity of NF-κB in the liver [3]. NF-κB was induced when reperfusion disturbance occurred in the liver transplantation [4, 42, 43]. However, NF-κB activation is reported to have a protective effect on liver grafts from heart beating donors [5]. However, no study has investigated the mechanism of reperfusion injury in the liver grafts from cardiac arrest donors, and this study is the first report clearly indicating a relationship between TNFα, NF-κB activity, and Kupffer cells in reperfusion injury of the liver under agonal conditions.

NF-κB is composed of heterodimers p65 and p50. We conducted a supershift assay of NF-κB and found that NF-κB, which was induced by preservation/reperfusion injury, consisted of p50 homodimer. KBF-1 is identified as a transcription factor of the p50 homodimer which binds to the near NF-κB consensus site of DNA existing in the promoter region of MHC class I gene [17]. Therefore KBF-1 is likely to have a function similar to that of NF-κB [17]. It remains to be clarified whether the enhancement of the activity of transcription factors directly causes the deterioration of the liver graft after reperfusion. Therefore further studies are necessary to clarify the true mechanism.

As immediate early genes, there are AP-1, signal transducers and activators transcription-3, NF IL-6 (C/EBP), and cyclic AMP response element binding protein as well as NF-κB. These components are strongly associated with signal transduction of acute-phase inflammatory reactions. This study identified an absence of NF-κB and AP-1 activities during cold preservation despite agonal conditions, but their activities increased during reperfusion after cold preservation. Significant buildup of NF-κB and AP-1 activities were recognized especially in the liver specimens from the cardiac arrest model under agonal conditions. However, this

phenomenon was not induced by Kupffer elimination. These results indicate that inhibition of the enhancement of NF-κB and AP-1 activities was strongly associated with prevention of preservation/reperfusion injury in the liver graft with an agonal condition, and that this activation of NF-κB binding and AP-1 was induced by inflammatory cytokines, such as TNFα and IL-1β produced by Kupffer cells.

In conclusion, our study clearly indicates enhancement of TX production and inflammatory cytokines and that these changes in chemical mediators are strongly associated with sinusoidal microcirculatory disturbance if there are Kupffer cells in the liver. This ischemic/reperfusion study of the liver from an NHBD agonal rat clarified that the activities of transcription factors, i.e., NF-κB and AP-1 are increased after reperfusion, and that the elimination of Kupffer cells prevents the rise in these transcription factors and microcirculatory disturbances. In clinical transplantation the use of liver grafts from NHBD still remains a controversial issue; however, the control of Kupffer cell function must be the key to success for transplantation from NHBD.

References

1. Ballou LR, Chao CP, Holness MA, Barker SC, Raghow R (1992) Interleukin-1-mediated PGE2 production and sphingomyeline metabolism. Evidence for the regulation of cyclooxygenase gene expression by sphingosine and ceramide. J Biol Chem 267:20044–20050
2. Beg AA, Baltimore D (1996) An essential roll for NF-κB in preventing TNF-induced cell death. Science 274:782–784
3. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D (1995) Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. Nature 376:167–170
4. Bradham CA, Stachlewitz R, Gao W, Qian T, Jayadev S, Jenkins G, Hannun Y, Lemasters JJ, Thurmann RG, Brenner DA (1997) Reperfusion after liver transplantation in rats differentially activates the mitogen-activated protein kinases. Hepatology 25:1128–1135
5. Bradham CA, Schemmer P, Stachlewitz RF, Thurmann RG, Brenner DA (1999) Activation of nuclear factor-B during orthotopic liver transplantation in rats is protective and does not require Kupffer cells. Liver Transpl Surg 5: 282–293
6. Bremer C, Bradford BU, Hunt KJ, Kencht KT, Connor HD, Mason RP, Thurman RG (1994) Role of Kupffer cells in the pathogenesis of hepatic reperfusion injury. Am J Physiol 267:G630–G636
7. Caldwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ (1991) Kupffer cell activation and endothelial cell damage after storage of rat livers: effect of reperfusion. Hepatology 13:83–95
8. Casavilla A, Ramirez C, Shapiro R, Nghiem D, Miracle K, Bronsther O, Randhawa P, Broznick B, Fung JJ, Starzl T (1995) Experience with liver and kidney allografts from non-heart-beating donors. Transplantation 59:197–203
9. Caughey GE, Pouliot M, Cleland LG, James MJ (1997) Regulation of tumor necrosis factor-alpha and IL-1 beta synthesis by thromboxane A2 in non adherent human monocytes. J Immunol 158:351–358
10. Clavien PA, Harvey PRC, Strasberg SM (1992) Prevention and reperfusion injuries in liver allografts. Transplantation 53:957–978
11. Dignam JD, Lebovitz RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489
12. Fukuzawa K, Emre S, Senyuz O, Acarli K, Schwartz ME, Miller CM (1995) N-Acetylcysteine ameliorates reperfusion injury after warm hepatic ischemia. Transplantation 59:6–9
13. Gasbarrini A, Borle AB, Farghali H, Bender C, Francavilla A, Van Thiel D (1992) Effect of anoxia on intracellular ATP, Na^{+}, Ca^{2+}, Mg^{2+} and cytotoxicity in rat hepatocytes. J Biol Chem 267:6654–6663
14. Gores GJ, Kost LJ, LaRusso NF (1985) The isolated perfused rat liver: conceptual and practical considerations. Hepatology 6:511–517
15. Inoue T, Yoshida Y, Nishimura M, Kurosawa K, Tagawa K (1993) Ca^{2+}-induced, phospholipase-independent injury during reoxygenation of anoxic mitochondria. Biochim Biophys Acta 1140:313–320
16. Kanno M, Ohkohchi N, Terasima T, Seya K, Mori S, Inaba H (1993) Lipid peroxidation of parenchymal hepatocytes during cold preservation and after reoxygenation in rats. Transplant Proc 25:2716–2721
17. Kieran M, Blank V, Logeat F, Vandekerckhove J, Lottspeich F, Le Bail O, Urban MB, Kourilsky P, Baeuerle PA, Israel A (1990) The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the rel oncogene product. Cell 62:1007–1018
18. Kurihara T, Akimoto M, Kurokawa K, Ishigro H, Niimi A, Maeda A, Shigemoto M (1992) Relationship between endothelin and thromboxane A2 in rat microcirculation. Life Sci 51:1281–1285
19. Marubayashi S, Takenaka M, Dohi K, Ezaki H, Kawasaki T (1980) Adenine nucleotide metabolism during hepatic ischemia and subsequent blood reflow period and its relation to organ viability. Transplantation 30:294–296
20. Melemdz HV, Heaton ND (1999) Understanding "marginal" liver grafts. Transplantation 68:469–471
21. Nakamura H, Nemenoff RA, Gronich JH, Bonventre JV (1991) Subcellular characteristics of phospholipaseA2 activity in rat kidney. Enhanced cytosolic, mitochondrial, and microsomal phospholipase A2 enzymatic activity after renal ischemia and reperfusion. J Clin Invest 87:1810–1818
22. Nathan HM, Jarrell BE, Broznik R, Kochik R, Hamilton B, Stuart S, Ackroyd T, Nell M (1991) Elimination and characterization of the potential renal organ donor pool in Pennsylvania, Transplantation 51:142–149
23. Ohkohchi N, Sakurada M, Endoh T, Koyamada N, Katoh H, Koizumi M, Orii T, Satomi S, Taguchi Y, Mori S (1991) Role of free radicals and energy synthesis on primary graft nonfunction in liver transplantation. Transplant Proc 23:2416–2419
24. Ohkohchi N, Sakurada M, Endoh T, Satomi S, Taguchi Y, Mori S, Watanabe N (1993) Electron leakage from mitochondria and lipid peroxidation after cold ischemia of liver. Int Hepatol Com 1:61–64
25. Ohkohchi N, Endoh T, Oikawa K, Seya K, Satomi S (1999) Fragility of the electron transport chain and superoxide generation in mitochondria of the liver graft after cold ischemia. Transplantation 67:1173–1177

26. Oikawa K, Ohkohchi N, Sato M, Satomi S (2000) The effect of elimination of Kupffer cells in the isolated perfused liver from non-heart-beating rat. Transpl Int 13 [Suppl 1]:S573–S579
27. Post S, Goerig M, Otto G, Manner M, Foltis C, Hofmann W, Herfarth C (1991) Rapid increase in the activity of enzymes of eicosanoid synthesis in hepatic and extra hepatic tissues after experimental liver transplantation. Transplantation 51:1058–1065
28. Samuelsson B, Goldyne M, Granstrom E, Hamberg M, Hammarstorm S, Malmsten C (1978) Prostaglandins and thromboxane. Annu Rev Biochem 47:997–1029
29. Sen R, Baltimore D (1986) Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 46:705–716
30. Shibuya H, Ohkohchi N, Seya K, Satomi S (1997) Kupffer cells generate superoxide anions and modulate reperfusion injury in rat livers after cold preservation. Hepatology 25:356–360
31. Shibuya H, Ohkohchi N, Tsukamoto S, Satomi S (1997) Tumor necrosis factor-induced, superoxide-mediated neutrophils accumulation in cold ischemic/reperfused rat liver. Hepatology 26:113–120
32. Soejima Y, Yanaga K, Nishizaki T, Yoshizumi T, Uchiyama H, Sugimachi K (1998) Effect of thromboxane synthetase inhibitor on non-heart-beating donors in rat orthotopic liver transplantation. Surgery 123:67–72
33. Takahashi Y, Ganster RW, Ishikawa T, Okuda T, Gambotto A, Shao L, Murase N, Geller DA (2001) Protective role of NF-κB in liver cold ischemia/reperfusion injury: effects of IκB gene therapy. Transplant Proc 33:602
34. Terashima T, Ohkohchi N, Kanno M, Orii T, Satomi S, Taguchi Y, Mori S (1993) Lipid per oxidation of parenchymal and non-parenchymal hepatocytes during cold preservation and after reoxygenation in rats. Transplant Proc 25:1678–1680
35. Terashima T, Ohkohchi N, Kanno M, Seya K, Satomi S, Taguchi Y, Mori S (1996) Role of neutrophils in lipid peroxidation at reperfusion in liver transplantation. Transplant Proc 28:324–326
36. Thurman RG, Lindert KA, Cowper KB, te Koppele JM, Currin RT, Caldwell-Konnel J, Takaya Y, Gao W, Takei Y, Marzi I, Lemasters JJ Activation of Kupffer cells following liver transplantation. In: Wisse E, Knook DL, McCuskey RS (eds) Cells of the hepatic sinusoid vol 3. Kupffer cell Foundation, Linden, pp 358–363
37. Tsukamoto S, Ohkohchi N, Fukumori T, Orii T, Asakura T, Takayama J, Shibuya H, Katoh H, Satomi S (1999) Elimination of Kupffer cells and nafamostat mesilate rinse prevent reperfusion injury in liver grafts from agonal non-heart-beating donors. Transplantation 67:1396–1403
38. Van Rooijen N (1989) The liposome-mediated macrophage "suicide" technique. J Immunol Methods 124:1–6
39. Yokoyama I, Kobayashi T, Negita M, Hayashi S, Yasutomi M, Katayama, Uchida K, Takagi H (1996) Liberation of vasoactive substances and its prevention with thromboxane A2 synthase inhibitor in pig liver transplantation. Transpl Int 9:76–81
40. Yokoyama I, Negita M, Kobayashi T, Hayashi S, Hachisuka T, Sato E, Orihata A, et al (1996) Beneficial effect of donor pretreatment with thromboxane A2 synthase inhibitor on the graft survival in pig liver transplantation. J Surg Res 60:232–238
41. Zhang Y, Ohkohchi N, Oikawa K, Sasaki K, Satomi S (2000) Assessment of viability of the liver graft in different cardiac arrest models. Transplant Proc 32:2345–2347
42. Zwacka R, Zhang Y, Zhou W, Halldorson J, Engelhardt JF (1998) Ischemia/reperfusion injury in the liver of BALB/c mice activates AP-1 and nuclear factor B independently of IB degradation. Hepatology 28:1022–1030
43. Zwacka R, Zhou W, Zhang Y, Darby CJ, Dudus L, Halldorson J, Engelhardt JF (1998) Redox gene therapy for ischemia/reperfusion injury of the liver reduces AP1 and NF-κB activation. Nat Med 4:698–704

ELSEVIER

Elimination of Kupffer Cells Suppresses Activation of Nuclear Factor Kappa B and Production of Cytokines and Eicosanoids in Non-Heart-Beating Donors

K. Oikawa, N. Ohkohchi, M. Sato, A. Masamune, and S. Satomi

THE SHORTAGE of donors has become a serious problem in liver transplantation (LTx). In recent years, to resolve this problem, LTx of the graft from non-heart-beating donors (NHBD) has become a topic all over the world. But liver grafts from NHBD have been considered unsuitable for LTx because the graft viability and sinusoidal microcirculation are deteriorated by warm ischemia and severe reperfusion injury.[1,2] Many studies have been carried out to clarify the mechanisms of reperfusion injury. Recent studies showed that Kupffer cells activated at reperfusion and they played important roles in reperfusion injury.[3] It is likely that many chemical mediators, that is, cytokines, eicosanoids, and reactive oxygen intermediates, would be produced from Kupffer cells. Furthermore in recent years, it has been reported that nuclear factors, for example, nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1), are very important factors in the biologic reactions. The purpose of this study is to clarify the behavior of nuclear factors, cytokines, and eicosanoids at the reperfusion of the liver graft and the effect of elimination of Kupffer cells on the reperfusion injury in NHBD.

MATERIALS AND METHODS

Animals and Protocol of Examination

Male Wistar rats, weighting 240 to 300 g, were allocated to four groups. In the control group ($n = 6$), liver was harvested under heart beating and perfused without cold preservation. In the heart beating (HB) group ($n = 6$), liver was harvested under the heart beating and perfused 6 hours after cold preservation in University of Wisconsin (UW) solution. In the non-heart-beating (NHB) group ($n = 6$), the cardiac arrest was induced by thoracotomy through the diaphragm, and 30 minutes after thoracotomy, livers were harvested and perfused after 6 hours of cold preservation in UW solution. In the Kupffer cell eliminated (KE) group ($n = 6$), rats were administered 5 mL/body of liposome-encapsulated dichloromethylene diphosphonate (0.47 mg in phosphate-buffered saline) intravenously to eliminated Kupffer cells 42 hours before the graftectomy.[4] Under anesthesia with 50 mg/kg of pentobarbital sodium intraperitoneally, the liver was flushed with 10 mL of 4°C saline and 20 mL of 4°C UW solution via the portal vein. In the HB, NHB, and KE groups the liver graft was preserved in UW solution at 4°C for 6 hours. The liver was perfused for 60 minutes at a pressure of 10 cmH$_2$O with 100 mL of Krebs-Henseleit bicarbonate buffer (pH 7.4, at 37°C) saturated with gas mixture of 95% O_2 and 5% CO_2 in the recirculating system as described previously.[5]

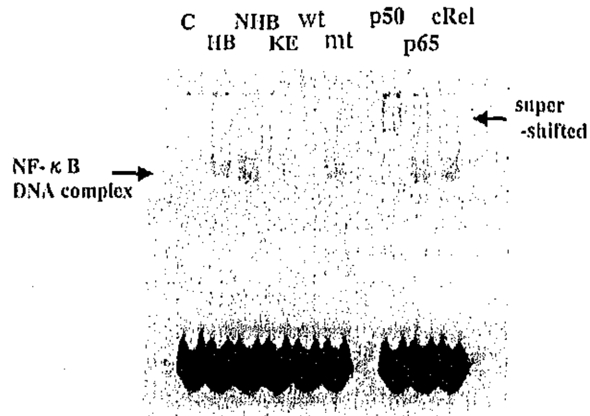


Fig 1. EMSA for activated NF-κB 1 hour after reperfusion. Lanes 5 to 9 show the competition and supershift assays of the NHB group. The band of NHB could be competed by 100-fold excess of unlabeled wild type oligonucleotide (lane 5), but it could not be competed with a mutated form (lane 6). Super shift assays with antibodies against p50, p65, and c-Rel clarified the identity of NF-κB (lanes 7 to 9). By this assay, it was revealed that p50/p50 homodimer was dominant.

Nuclear Factor DNA Binding Activity

Nuclear extracts were prepared from frozen liver specimen and electrophoretic mobility assay (EMSA) was performed.

Cytokines and Eicosanoids

Tumor necrosis factor α (TNF-α), interleukin-1 β (IL-1β), thromboxaneB2(TXB2), 6-ketoProstaglandinF1 α(6-ketoPGF1 α), and leukotrieneB4(LTB4) were measured in the perfusate of the liver using commercially available enzyme-linked immunosorbent assay kits.

From the Division of Advanced Surgical Science and Technology and the Division of Gastro-Intestinal Disease, Graduate School of Medicine, Tohoku University, Sendai, Japan.

Address reprint requests to N. Ohkohchi, the Division of Advanced Surgical Science and Technology, Graduate School of Medicine, Tohoku University, 1-1 Seiryo-machi, Aoba-ku, Sendai, Japan.

© 2001 by Elsevier Science Inc.
655 Avenue of the Americas, New York, NY 10010

0041-1345/01/$-see front matter
PII S0041-1345(00)02339-3

Table 1. Cytokines and Eicosanoids Released From the Perfused Liver

	Control	HB	NHB	KE
IL-1β (pg/mL)	34.2 ± 13.0	32.8 ± 12.2	61.4 ± 14.7*	40.6 ± 18.5
TNFα (pg/mL)	0.3 ± 0.1	4.5 ± 6.3	3.2 ± 5.1	0.4 ± 0.6
TXB2 (pg/mL)	81.3 ± 5.6	2017 ± 1525	2762 ± 1380	16.7 ± 18.3†
6-ketoPGF1α (pg/mL)	178 ± 71.8	346 ± 119‡	150 ± 29.5	120 ± 59.1
TXB2 to 6-keto PGF1 α ratio	0.42 ± 0.1	4.32 ± 2.30	18.6 ± 9.6	0.1 ± 0.1§

*$P < .05$ vs KE, $P < .01$ vs control and HB.
†$P < .01$ vs HB and NHB.
‡$P < .05$ vs control, HB, and KE.
§$P < .05$ vs HB, $P < .01$ vs HNB.

Histologic Examination

Light microscopical findings were investigated. Specimens were stained with hematoxylin and eosin.

Statistical Analysis

All values are presented the mean ± standard deviation. Statistical analysis was performed using Student's *t* test. $P < .05$ was considered as statistically significant.

RESULTS

Nuclear Factor DNA Binding Activity

NF-κB activity in the NHB group was higher than in other groups. In the KE group, the activity value was suppressed at the same level as in the HB group (Fig 1). Examination results of AP-1 activity was the same as that of NF-κB activity (data not shown).

Cytokines in Perfusate

IL-1β. In the NHB group, the concentration of IL-1β was significantly higher than in the other groups. In the KE group, the production of IL-1β was suppressed to the same level as the control group.

TNFα. In the KE group, the production of TNFα was suppressed to the same level as the control group, but there was no significant difference between each group (Table 1).

Eicosanoids in Perfusate

TXB2. In the NHB group, the concentration of TXB2 was significantly higher than in the other groups. In the KE group, the concentration of TXB2 was completely suppressed.

6-keto PGF1 α. In the HB group, the concentration of 6-keto PGF1α was significantly higher than in the other groups. In the NHB group, the concentration was reduced to the same level as in the control group.

TXB2 to 6-keto PGF1α ratio. In the NHB group, the ratio was significantly larger than in the other groups. In the KE group, the ratio was completely suppressed compared with those in the HB and NHB groups.

LTB4. LTB4 could not be detected in any groups (Table 1).

Histological Examination

In the NHB group, sinusoidal space became narrow, and hydropic changes in hepatocytes were observed. But in the KE group, sinusoidal structures and hepatocytes were well preserved, as in the control group.

DISCUSSION

The shortage of donors in LTx has become a serious problem all over the world. It is generally accepted that liver can be transplanted only when harvested prior to cardiac arrest because warm ischemia causes liver damage and eventually graft failure. In some countries the LTx from NHBD has been carried out, but the result of LTx is not satisfactory. In the LTx from agonal NHBD, donors have unstable condition and various clinical courses leading to the cardiac arrest. It is still unclear why the transplantations of liver grafts from agonal NHBD has been unsuccessful. We reported that Kupffer cells generate oxygen free radicals and release cytokines at reperfusion after cold ischemia and modulate the degree of injury of sinusoidal endothels.[6] And in recent years, many biologic phenomena have been explained by the change of a gene by the technique of molecular biology. Nuclear factors are very important substrates in the biologic reactions. We clarified that the microcirculatory disturbance after LTx from agonal NHBD was the main cause, followed by the deterioration of sinusoidal endothelial cells and hepatocytes caused by activated Kupffer cells.[1] In our present study, elimination of Kupffer cells suppressed the activation of nuclear factors NF-κB and AP-1 and the production of TXB2 and cytokines. Furthermore ischemia/reperfusion injury of the sinusoidal microcirculation and hepatocytes was prevented by elimination of Kupffer in cells NHBD. Our results indicate that Kupffer cells play an important role in the mechanism of graft injury in LTx from NHBD and that LTx using grafts from NHBD will be succeeded by modulation of Kupffer cells.

REFERENCES

1. Tsukamoto S, Ohkohchi N, Fukumori T, et al: Transplantation 67:1396, 1999
2. Ohkohchi N, Tsukamoto S, Endoh T, et al: Organ Tissues 1:13, 1999
3. Caldwell-Kenkel JC, Currin RT, Tanaka Y, et al: Hepatology 13:83, 1991
4. Van Rooijen NL: J Immunol Methods 124:1, 1989
5. Gores GJ, Kost LJ, LaRusso NF: Hepatology 6:511, 1985
6. Shyibuya H, Ohkohchi N, Seya K, et al: Hepatology 25:356, 1997

課題３：死戦期を伴う心停止ドナー肝の冷保存・再潅流障害に対する薬剤の投与効果の検討

1. 要約

【目的】近年肝移植におけるドナー不足は深刻さを増し、心停止ドナーからの肝移植も試みられてはいるが、初期グラフト機能不全を高率に引き起こすため現段階では不適当とされている。本研究の目的は、心停止ドナー肝移植の成功に向け、p38 MAPK 抑制作用をもつ選択的 TNF-α,IL-1β産生阻害剤 FR167653 及び強力なセリンプロテアーゼインヒビターである nafamostat mesilate の温阻血再灌流障害における効果を明らかにするとともに、屍体肝グラフトのコンディショニング法を開発することである。

【方法】雄性 Wistar rat を使用し、5群に分け肝摘出再灌流実験を施行した。1)Heart beating 群(HB 群)：心拍動下に肝摘出。6 時間冷保存後再灌流。2)Non-heart-beating 群(NHB 群)：呼吸停止から心停止を誘導し、心停止下に肝摘出。6時間冷保存後再灌流。3)nafamostat mesilate 投与群(NM 群)：nafamostat mesilate 投与後,NHB 群と同様に肝摘出。冷保存後再灌流。4)FR167653 投与群(FR 群)：FR167653 投与後,NHB 群と同様に肝摘出。冷保存後再灌流。4)FR167653+ nafamostat mesilate 投与群(FR+NM 群)：nafamostat mesilate 及び FR167653 投与後,NHB 群と同様に肝摘出。冷保存後再灌流。5 群とも灌流時間は 1 時間としその後にサンプルを採取した。検査項目は、胆汁産生量、灌流液量、生化学検査、IL-1β,TNF-α, thromboxaneB2(TXB2), 6-ketoPGF1α, leukotrieneB4, Cox-2, NF-κB, AP-1, 病理組織(電顕、光顕)である。

【結果】HB 群と比し、NHB 群で IL-1β,TNF-α,NF-κB, AP-1, TXB2, leukotrieneB4, Cox-2 が有意に高く、FR+NM 群では NHB 群に比し有意に低下した。病理所見でも FR, FR+NM 群で肝類洞の微小循環障害が改善された。以上の結果から、FR167653 と nafamostat mesilate は、温阻血再灌流障害時において、炎症性サイ

トカイン及びアラキドン酸カスケードのメディエーターを著明に抑制することが確認された。また、この 2 剤併用療法は、屍体肝グラフトの肝類洞における微小循環保持に極めて有用であることが示唆された。

【結語】心停止ドナーからの肝移植では、死戦期（温阻血期）における IL-1β・TNF-αの抑制およびアラキドン酸カスケードの抑制が重要であると考えられた。FR167653 と nafamostat mesilate の 2 剤併用療法は、屍体肝グラフトのコンディショニング法として効果があると考えられた。

2. 研究背景

現在肝移植は、先天的・後天的肝疾患における肝不全に対する治療方法として確立され、既に標準的な治療法となってきた。日本においても脳死移植法が制定され、脳死移植が平成 9 年より開始されている。しかしながら臓器提供者（以下ドナーと記す。）の数には限りがあり、ドナー不足は全世界的に問題となってきている。そこで近年、いわゆるマージナルドナーの利用が検討され[1]、心臓停止ドナーからの肝移植の可能性についても検討されている。いくつかの施設では既に心停止ドナー肝移植を試みてはいるが[2,3]、その結果は芳しくないのが実情である。とくにuncontrolled non-heart beating donor: uncontrolled NHBDからの肝グラフトは、初期グラフト機能不全（primary graft non-function: PGN）が高頻度に生じるため現段階では不適当とされている[2,3]。

肝移植において保存肝の虚血再灌流障害は PGN の大きな要因と考えられ[4]、このメカニズムを解明するため多くの研究がなされている。そして、この障害発生機序には類洞内皮細胞の障害やクッパー細胞の活性化による微小循環障害が大きく関与しているとの報告がなされている[5,6,7]。当教室でもこれまで冷保

存再灌流障害の際の活性酸素産生、クッパー細胞の活性化、TNF-αの放出、好中球の関与等について報告してきた[8,9]。さらに心停止肝移植では、低酸素血症、低血圧、電解質異常、アシドーシス等を伴ういわゆる死戦期を経た温阻血が加わり、冷保存再灌流障害と比較しても、より複雑な生体反応が生じることが知られている[10]。これらの研究結果に基づき、当教室のTsukamotoらは、クッパー細胞除去を目的としたジスクロロメチレンジフォスフォネート(liposome-encapsulated dischloromethylene diphosphonate: LD)を使用し、ブタ屍体肝移植を施行した[10]。そして、短時間の冷保存下においては屍体からの肝移植に成功し、Kuppfer細胞を除去することで温阻血再灌流障害を軽減できることを報告した。また、同じく当教室のShibuyaらはTNF-αやフリーラジカルを抑制することにより虚血再灌流障害を軽減できることを報告した[11]。これにより、Kuppfer細胞の活性化を抑え、TNF-αやフリーラジカルの産生を抑制することで虚血再灌流障害を軽減できることが確認できた。しかしKuppfer細胞を除去する薬物LDは、それ自体の強力な毒性のため臨床的には使用することができない。これらのことから、近年開発された選択的IL-1β・TNF-α産生阻害剤FR167653[12](Figure 1)が温阻血再灌流障害に対して効果的ではないかと考えた。FR167653はp38 MAPKのpathwayを抑制することでAP1系の核内転写を抑制し、炎症性サイトカイン、特にIL-1β・TNF-αの産生を選択的に阻害することが知られている[12]。さらに温阻血再灌流障害では、及川らの報告にもあるように肝微小循環障害の改善が重要な鍵となる[13]。このため、強力なセリンプロテアーゼ阻害剤でありDICの治療薬として知られているnafamostat mesilateが効果的ではないかと考えた。つまり抗炎症作用を期待されたのがFR167653、微小循環障害改善作用を期待されたのがnafamostat mesilateである。そしてこの2剤の併用により、サイトカインの大量放出や類洞内皮細胞の障害、クッパー細胞の活性化、さらにア

ラキドン酸カスケードの活性化を抑制することができ、屍体肝グラフトの微小循環障害を軽減できるのではないかと考えた。

3. 研究目的

本研究の目的は、心停止肝移植の成功に向け、選択的 TNF-α・IL-1β産生阻害剤である FR167653 および強力なセリンプロテアーゼインヒビターである nafamostat mesilate の温阻血再灌流障害における効果を明らかにするとともに、新しい屍体肝グラフトのコンディショニング方法を開発することである。

4. 研究方法

1. 実験動物

この動物実験はすべて、米国の National Institutes of Health により出版された "Guide for the Care and Use of Laboratory Animals," に沿って施行された。

２８０−３４０g の雄性 Wistar ラット（日本チャールズ・リバー）を以下の５群に分け、摘出肝の常温酸素化灌流を施行した[14]。1)Heart beating 群(HB 群)：心拍動下に肝を摘出し６時間の冷保存後再灌流した群。2)Non-heart-beating 群(NHB 群)：呼吸停止から心停止を誘導し、30 分の温阻血時間を置いた後、心停止下に肝摘出。6 時間冷保存し再灌流。3)nafamostat mesilate 投与群(NM 群)：nafamostat mesilate を 0.2 mg/kg/hour で 30 分間持続静注後、呼吸停止から心停止を誘導。その後は NHB 群と同様に肝摘出、冷保存後再灌流。4)FR167653 投与群(FR 群)： FR167653 を 2mg/kg ゆっくり静注し、5 分後に呼吸停止から心停止を誘導。

その後は NHB 群と同様に肝摘出、冷保存後再灌流。5)FR167653+ nafamostat mesilate 投与群(FR+NM 群)：nafamostat mesilate 及び FR167653 を NM・FR 群と同様に投与後、NHB 群と同様に心停止下肝摘出。冷保存後再灌流。5 群とも灌流時間は 1 時間としその後にサンプルを採取した。それぞれ n＝5 とした。

2. 肝摘出法

ジエチルエーテル吸入による全身麻酔後、全身ヘパリン化(heparin 1000 unites/kg, 静脈内投与)を行なった。正中切開により開腹。総胆管にシリコンチューブを挿入後、門脈に 12G サーフロー針外套を cut down 法で挿入した。下大静脈切開後、約 10 cmH_2O の圧で 4 ℃生理食塩水 20ml、4 ℃ University of Wisconsin solution (UW 液) 20 ml を用い門脈血を wash out した。全肝を摘出し、4℃ UW 液にて単純浸漬保存した。

3. 心停止モデルの作製

全身麻酔、全身ヘパリン化後、心窩部正中に約 2cm の皮切をおき、総胆管にシリコンチューブを挿入後、経腹腔的に横隔膜を切開、呼吸停止から心停止を誘導した。心停止までの時間は表に示すとおりで、各群間に有意な差は認めなかった（Table 1）。心停止後３０分間室温に放置した後に、前記肝摘出法と同様に門脈血を wash out し、肝を摘出、4 ℃ UW 液にて単純浸漬保存した。

4. 灌流方法

Gores らの回路[14]（Figure 2）を使用した。まず回路を 0.5%クロルヘキシジン液にて 30 分間循環消毒し、0.2 μm filter を通した蒸留水で洗浄した。この回路に Krebs-Henseleit bicarbonate buffer を入れ 95% O_2 / 5% CO_2 混合ガスで飽和した。この buffer で、肝を 60 分間、37℃、10cmH_2O にて常温定圧酸素化灌流した。

5. 検討項目

1）．胆汁産生量、門脈灌流液量

灌流した60分間に総胆管から流出した胆汁を回収し、ラット体重当たりの時間胆汁産生量を求めた。また、灌流60分間に肝静脈からの流出した灌流液を回収し、ラット体重当たりの門脈灌流液量を求めた。

2）．生化学的検査

灌流 60 分後の灌流液中、aspartate aminotransferase (AST)、alanine aminotransferase (ALT)、lactate dehydrogenase (LDH)を酵素比色法にて測定した。(AST, ALT: Iatron Laboratories, Inc., Tokyo) (LDH: Wako Pure Chemical Industries, Osaka)

3）．炎症性サイトカイン

灌流 60 分後の灌流液中、interleukine-1 β (IL-1 β)、tumor necrosis factor α (TNF-α)を enzyme-linked immunosorbent assay kits (BioSource International, Inc., Camarillo, CA)にて測定した。

4）．各種転写因子活性の検討

60分間灌流後の nuclear factor kappa B (NF-κB)、activating protein 1 (AP-1)の DNA binding activity を electrophoretic mobility shift assay (EMSA)[13,15]にて検討した。

a) 組織からの核蛋白抽出法

肝組織は液体窒素を用いてfreezed clump し、-80℃で保存した。核蛋白抽出のため約500mg の凍結標本を液体窒素の入った乳鉢で粉末にした。核蛋白抽出はDignamらの方法[16]に準じ、以下の操作はすべて氷上で、また遠心は0℃にて行った。溶液A (0.6% IGEPAL CA-630, 150mM NaCl, 10mM HEPES pH7.9, 1mM EDTA, 0.5mM phenylmethylsulufonyl fluoride(PMSF)) 5mlに粉末状にした標本を溶かし、15ml Dounce tissue homogenizer (Wheaton, Millville,NJ) でpestle B を用い10回程度穏やかにホモジナイズした。その後破壊されていない組織を除くため、

ホモジナイズ液すべてを15mlのFalcon チューブにとり2000rpmで1分間遠心を行った。上清を氷上に 5 分間おき、その後5000rpmで 5分間遠心し、核を分離した。沈殿した核を溶液B(25%glycerol, 20mM HEPES pH7.9, 420mM NaCl, 1.2 MMgCl2, 0.2mM EDTA, 0.5mM dithiothreitol, 0.5mMPMSF, 2mM benzmidine, 5μg/mL pepstatin, 5μg/mL leupeptin, 5μg/mL aprotinin)に移し氷上で20分間置いた。融解した核を microcentrifuge tube に入れ遠心し、核蛋白を分離した。上清を分注後、液体窒素で凍結し-80℃で保存した。また、核蛋白量はBCA protein assay(Pierce, Rockford, IL) にて定量した。

b) Electrophoretic Mobility Shift Assay (EMSA)

Double-stranded oligonucleotide (NF-κB: 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (Promega, Madison, WI), AP-1: 5'-CGC TTG ATG AGT CAG CCG GAA-3', (Promega))に T4 polynucleotide kinase(Promega)を用いて、γ-^{32}P ATP をラベルした (37℃で 30 分間インキュベート)。その後 Quick Spin G-25 column (Boehringer Mannheim) にて purification し、probe とした。核抽出液(タンパク量として 15μg)と probe を binding reaction buffer(10mM Tris-Cl pH7.5, 1mM MgCl2, 50mM NaCl, 0.5 mM EDTA, 0.5mM dithiothreitol, 0.5mM PMSF, 4% glycerol, 0.1 mg/ml poly(dI.dC)-poly(dI.dC))を用い室温にて 20 分間インキュベーションした。それらを TBE buffer 中の 4%polyacrylamide gel に流し、150V で 2 時間泳動した。ゲルドライ後 -80℃でフィルムに感光させた。competition assay では、核蛋白と100 倍量の無ラベル oligonucleotide 及び mutant oligonucleotide を、それぞれ probe を入れる前に室温 で 10 分間インキュベートし、同様に assay を行なった。また、NF-κBに関してはprobe を入れる前に抗p50、p65、c-Rel 抗体(2μg/sample, Santa Cruz Biotechnology,Inc) を用い 4 ℃で 1 時間インキュベートし super shift assay を施行した。

5）．Cyclooxgenase-2 (Cox-2)

アラキドン酸カスケードの key enzyme である Cox-2 が TXB2 や 6-ketoPGF1α の産生に関与するため、Cox-2 の m-RNA 量を Northern blotting 法にて測定した。

Cox-2 の complementary DNA (cDNA) プローブは pGEM-T Easy vector (Promega, Madison, WI)を使用してクローニングした。 そして Cox-2 プライマー (5'-CCCAGCACTTCACTCATCAG-3'、5'-GCGGATGCCAGTGATAGAGT-3')を使用したシークエンスにて塩基配列を決定し、Cox-2 のプローブとして間違いのないことを確認した。

Northern blotting 法については下記に述べるとおりである。The acid guanidinium thiocyanate-phenol chloroform method[17]により抽出した等量の RNA を 1.2%(wt/vol) アガロースフォルムアルデヒドゲルにて電気泳動し、Hybond-XL ナイロンメンブレン(Amersham International, Amersham, England)に転写した。そして UV-linker にてメンブレンを固定した。Cox-2 の c-DNA probe は、BcaBEST DNA labeling kit (Takara, Tokyo) を使用し alpha ^{32}P deoxycytidine triphosphate でラベルリングした。42°C のハイブリダイゼーションバッファー｛50% (vol/vol) formamide, 5x SSPE [(in mmol/L): NaCl, 150; NaH_2PO_4, 10; and ethylenediamine-tetraacetic acid, 1; pH 7.0], 2x Denhardt's solution (Wako Pure Chemical Industries), 0.5% (wt/vol) sodium dodecyl sulfate (SDS), 100 μg/ml denatured salmon sperm DNA｝内で、ラベルしたプローブをメンブレンと 20 時間以上ハイブリダイゼーションさせた。その後 42℃定温下で、洗浄液 A ｛2x SSPE containing 0.5% (wt/vol) SDS｝を使用し、メンブレンを 30 分間×2 回洗浄、さらに洗浄液 B｛0.2x SSPE containing 0.1% (wt/vol) SDS ｝を使用し 30 分間×2 回洗浄した。洗浄したメンブレンは、Fuji radiograph films (Fuji photo Film Co., LTD., Tokyo) を使用してオートラジオグラフィーにて分析した。コントロールとしては、ラット glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) cDNA を使用した。

6）．アラキドン酸カスケード産物

ThromboxaneB2(TXB2)などのアラキドン酸カスケード産物は、類洞の狭小化や類洞内凝固、微小循環障害等に関与することが知られている。このため、灌流 60 分後の灌流液中 TXB2、6-ketoProstaglandine α （6-ketoPGF1 α）、LeukotrieneB4 (LTB4)を enzyme-linked immunosorbent assay kits (Cayman Chemical Company, Ann Arbor, MI)にて測定した。

7）．光学顕微鏡所見

灌流 60 分後の各組織を 10%中性ホルマリン固定し、ヘマトキシリン-エオジン染色で観察した。

8）．電子顕微鏡所見

灌流 60 分後の各組織片を 0.1 M sodium cacodylate buffer (pH 7.3)中に混合したの 2.5%グルタルアルデヒドと 2%パラフォルムアルデヒドで前固定した。さらに 1% osmium tetroxide にて後固定したのち、段階的に濃度をあげたエタノールおよび無水アルコールに浸け脱水し、エポキシレジンに包埋した。包埋したサンプルはミクロトームで薄切し branyl acetate と lead citrate で染色、電子顕微鏡 (JEM-1010; Japan Electron Optics Laboratories, Tokyo)にて観察した。虚血再灌流時の類洞内皮細胞障害は Zone1 で強いことが知られているため[18]観察領域は Zone1 とした。

6. 統計学的検討

データは mean ± standard deviation (SD) で表した。また各群間の有意差については一元配置分散分析(One-way ANOVA)および多重比較検定(Scheffe's F)を施行し、 $P<0.05$ を有意差ありとした。

5. 研究結果

1. 胆汁産生量、門脈灌流液量

胆汁産生量は、HB 群・FR 群・FR+NM 群では、NHB 群に比し有意に高値を示した（Table 2）。

門脈灌流液量も、HB 群・NM 群・FR 群・FR+NM 群では、NHB 群に比し有意に高値を示した（Table 2）。

2. 生化学的検査

AST： HB 群・NM 群・FR 群・FR+NM 群では、NHB 群に比し有意に低値を示した（Table 2）。

LDH: HB 群・FR 群・FR+NM 群では、NHB 群に比し有意に低値を示した（Table 2）。

ALT：測定限界値以下であった。

3. 炎症性サイトカイン

IL-1β： HB 群・NM 群・FR 群・FR+NM 群では、NHB 群に比し有意に低値を示した（Figure 3）。

TNF-α： HB 群・FR 群・FR+NM 群では、NHB 群に比し有意に低値を示した（Figure 3）。

4. 転写因子活性

NF-κB： NHB 群で強い活性を認め、NM 群・FR 群・FR+NM 群では、活性が低下していた。competition assay においては、無標識の wild type oligonucleotide で競合阻害され、mutant type では競合されなかった。super shift assay では抗 p50・p65 抗体投与でバンドのシフトが認められた（Figure 4A）。

AP-1： NF-κB と同様に NHB 群で活性は強く、NM 群・FR 群・FR+NM 群で

は、活性が低下していた。competition assay においても、無標識の wild type oligonucleotide で競合阻害され、mutant type では競合されなかった (Figure 4B)。

5. Cox-2

NHB 群でのみバンドを認めた (Figure 5)。

6. アラキドン酸カスケード産物

TXB2： NHB 群で HB 群に比し有意に上昇したが、NM 群・FR 群・FR+NM 群では、NHB 群と比し有意に低下した。さらに、NM・FR+NM の両群は FR 群と比しても有意差は認めないが低下傾向にあった (Figure 6)。

6-keto PGF1α： NHB・NM・FR・FR+NM 群で、HB 群と比し有意に低下した (Figure 6)。

Leukotriene B4： NHB 及び FR の両群では、HB 群に比し有意に上昇したが、NM・FR+NM 群では、HB 群と同レベルであった (Figure 6)。

7. 光学顕微鏡所見

HB 群では、類洞腔が十分保たれており、肝移植直後に再灌流した状態に相当していた。肝細胞構造は良好に保たれていた (Figure 7A)。NHB 群では HB 群に比し類洞構造の破壊が著明で、類洞腔がほとんど消失している状態であった。また肝細胞も空胞状変性が広範囲に認められた (Figure 7B)。NM 群では、類洞腔は保たれているが、肝細胞の空胞状変性が広範囲に認められた (Figure 7C)。しかし、FR・FR+NM 群では HB 群と同等に組織学的構築が保たれていた (Figure 7D,7E)。

8. 電子顕微鏡所見

HB 群では、ほとんどの類洞内皮細胞がよく保たれており、Disse 腔も正常で、肝細胞の腫脹も認めなかった (Figure 8A)。しかし NHB 群では類洞内皮細胞の破壊が著明で、本来の類洞腔は不明瞭となっていた。また肝細胞の腫大化や、

ミトコンドリアの膨化も認められ、一部崩壊した肝細胞が類洞内に脱落する像が認められた（Figure 8B）。NM群では、類洞腔は保たれていたが、類洞内皮は多くの領域でその連続性の欠如を認めDisse腔の開大も多く認められた。肝細胞の腫脹は認めなかったが、胞体内には空胞状変性が散見された（Figure 8C）。FR群では、少数の肝細胞が崩壊していたが類洞腔は十分保たれており、多くの領域で類洞内皮細胞がよく保たれていた。しかし一部では類洞内皮の連続性欠如を認め、Disse腔の開大も認めた（Figure 8D）。FR+NM群では、HB群と同等に類洞腔が十分に保たれており、ほとんどの類洞内皮細胞はよく保たれていた。Disse腔の開大も認めず、肝細胞の腫脹も認めなかった（Figure 8E）。

6. 考察

現在ドナー不足は全世界的に問題となっており[1,19]、いくつかの施設では既に臨床の現場で心停止ドナー肝移植を施行している。しかし、その結果は芳しくなく、特にuncontrolled NHBDについてはいまだに不適当とされている[2,3,20,21]。心停止ドナー肝移植では、肝摘出前の温阻血が再灌流時の重篤な組織障害の原因となっていることが知られている[22,23]。以前我々は、TNF-αやIL-1βなどの炎症性サイトカインが冷保存後の再灌流時に上昇することを報告した[24]。そして肝移植時の虚血再灌流障害の主たる原因は微小循環障害であり、この微小循環障害は、クッパー細胞の活性化やTNF-α放出等により引き起こされた類洞内皮細胞障害や類洞内好中球の集積、類洞内凝固によるところが大きいことも既に報告している[11,13]。近年、p38 MAPKの抑制作用をもつTNF-α及びIL-1βの選択的産生阻害剤FR167653が開発された[12,25]。そこで今回我々は、このFR167653が温阻血再灌流障害においてもグラフトの抗炎症効果、さらには類洞内皮細胞障

害軽減といった効果を発揮するのか、そして強力なセリンプロテアーゼインヒビターである nafamostat mesilate を使用することで、心停止ドナー肝グラフトの微小循環障害の原因となる類洞内凝固やそれに引き続く類洞内皮細胞破壊といった現象を抑制できるのかを検討した。その結果、nafamostat mesilate と FR167653 はともに類洞構造の維持に効果的であることが確認できた。そして、特に 2 剤併用群（FR+NM 群）では、NM 単独群に比し胆汁産生量の有意な増加と TNF-α の更なる低下を認め、さらに FR 単独群と比しても門脈灌流液量の有意な増加や、IL-1β の更なる低下($p<0.05$)、電顕所見における類洞内皮細胞の保護効果も認められた。以上の結果より、この2剤併用療法が心停止ドナー肝グラフトのコンディショニングにおいて非常に効果的であることが解明された。

今回の実験で我々が使用したのは、呼吸停止から心停止を誘導したいわゆる死戦期を被った心停止モデルであった。このモデルを作成した理由は、これが uncontrolled NHBD の状態に最も近いからである。uncontrolled NHBD の細かい定義は各施設によって多少違いがあるのだが、基本的には血圧低下時に何も手が加わらずに（動静脈カニュレーションやそれに引き続く処置等をせずに）心停止したものを指す。したがって本研究ではこの基本に順じてカニュレーションのみならず昇圧剤や脱血等も一切施行しないモデルを作成したのである。uncontrolled NHBD から摘出したグラフトは、死戦期の期間中は半虚血状態の腸管からの門脈血を受けることになり、腸管虚血の影響や肝自体の鬱血による障害をかなり受けることとなる。腸管虚血は門脈血中のエンドトキシンレベルの上昇[26]や全身への TNF 放出[26]、そしてそれによる全身状態の悪化[27,28]を引き起こすことが知られている。しかし従来の KCL 注射や大動脈クランプによる心停止モデルは強制的心停止であり、腸管虚血の影響を全く受けないのである。そこで今回我々は、uncontrolled NHBD と同様に腸管虚血の影響を受ける様な、つ

まり死戦期を被った心停止モデルを作製し使用することとした。実際に我々は、このモデルと KCL による心停止モデルで、再灌流前の段階ではあるが ATPase 活性や TNF-αの値等において大きな違いがあることを以前報告している[29]。以上より、今後心停止ドナー移植を想定し温阻血再灌流障害の実験をする際には、このモデルを使うのが妥当だと我々は考えている。

NF-κB・AP-1 などの転写因子が炎症性サイトカインも含め多くの遺伝子を誘導すること、そして逆に、炎症性サイトカイン特に TNF-α・IL-1βといったところがNF-κB・AP-1 等の転写因子を誘導することも既によく知られている[30,31]。我々の研究ではNF-κB および AP-1 の活性化は、HB 群と比し、NHB 群でより強く認められ、nafamostat mesilate や FR167653 で抑制されることが解った。これらの結果から、NHB 群ではTNF-α・IL-1βといった炎症性サイトカインが活性化された NF-κB・AP-1 によって誘導され、その誘導された TNF-α・IL-1βが、再度 NF-κB・AP-1 の更なる活性化を誘導していると推測される。そのため、FR167653 により TNF-α・IL-1βが抑制されたとき、この悪循環が断ち切られ、NF-κB・AP-1 の活性化が抑制されたと考えられた。

細胞膜の重要な成分の 1 つであるアラキドン酸は、様々なストレス下で Cox や Lipoxygenas によって代謝されることが知られている[32,33]。Cox は Thromboxane や Prostacyclin の合成に関わっており、Cox-1 と Cox-2 が存在することが解っている[32,33]。Cox-1 はほぼすべての細胞に存在するが、Cox-2 は様々な状況で誘導される誘導蛋白である[34-38]。Shornick らは IL-1βノックアウトマウスを使用し Cox-2 が IL-1βによって誘導されることを示した[35]。また、Cox-2 が炎症および敗血症の状態において強く誘導されることや、エンドトキシンが Cox-2 を誘導するということも既に報告されている[36-38]。Cox-2 はそのプロモーター領域に NF-κB や AP-1 のドメインをもっている。このため Cox-2 は、NF-κB を介して

エンドトキシンや炎症性サイトカインにより誘導されると考えられている[37-40]。これらの見解からも、温阻血再灌流障害時にTNF-αやIL-1βを抑制することは、NF-κBやAP-1だけでなくCox-2やThromboxaneの合成を抑制することにつながり、グラフトのバイアビリティー改善にとって重要だと考えられる。もっとも、NF-κBは移植肝において保護的役割も持っているのだが[31]、過剰な活性化は、Thromboxaneの産生抑制や微小循環の維持という面からみても、障害になると考えられる。今回の結果では、Cox-2のバンドはNHB群でのみ認め、その他の群ではほとんど確認できないレベルであった。ラットでは、Cox-2は正常肝では確認できず、炎症時にのみ誘導されることが報告されている[37,38,41]。よって我々の研究では、NHB群においてのみNF-κBやAP-1のレベルが極めて高かったため、Cox-2のバンドが検出できたと考えらる。

以前より、肝グラフトの温阻血再灌流障害の主たる原因は肝実質障害であるとされてきた[2]。その機序としては、ATPの枯渇や細胞内カルシウムの上昇により最終的にフォスフォリパーゼAが活性化され細胞骨格や細胞膜の透過性に影響を及ぼすことなどが報告されている[42-46]。フォスフォリパーゼAはアラキドン酸カスケードの上流に位置する酵素で、TXA2やLeukotriene B4などはこのカスケードの産物である。すなわちTXA2の合成にはCoxだけでなくフォスフォリパーゼAも関与するのである[32,33]。TXA2はそれ自体強力な血管収縮作用や血小板凝集作用をもち[47,48]、血中では不安定な物質であるため最終的にTXB2となることが知られている。我々の研究では、NM、FR、FR+NM群でグラフトにおけるTXB2の産生を効果的に抑制することに成功した。これはFR167653による炎症性サイトカインや転写因子の抑制作用および、nafamostat mesilateによるフォスフォリパーゼA2抑制作用[33]によるものと考えられる。加えてFR+NM群では、電顕所見において、ほとんどの類洞内皮細胞とDisse腔がほぼ正常に近い状

態で保たれているのを確認できた。これらの結果からも、FR167653 と nafamostat mesilate の 2 剤併用は、Cox-2 の活性化及び TXB2 の合成を効果的に抑制することで再灌流時の類洞微小循環を維持し、虚血再灌流障害の軽減に効果を発揮することが強く示唆された。もっとも、なぜ NHB 群では TXB2 の合成のみが増加し、内皮細胞にとって保護的役割をする 6-keto PGF1 α の合成は増加しないのかという疑問は存在するだろう。6-keto PGF1 α もやはりフォスフォリパーゼ A や Cox により合成されるため TXB2 と平行して増加するはずである。しかし、及川らの報告[13]でもやはり温阻血をかけると TXB2 のみが増加し 6-keto PGF1 α は増加しなかったとなっている。原因としては、プロスタグランディンエンドペルオキシドから TXB2 や 6-keto PGF1 α が生成される過程で、温阻血自体に 6-keto PGF1 α の合成経路を抑制する刺激があるのではないかと推測されるが、現在のところ定かでない。考えられることとしては、PGI2 合成時、sensory neurons が活性化することで calcitonin gene-related peptide(CGRP)が放出され類洞内皮細胞での PGI2 合成が促進されることが知られているが、この経路で温阻血が何らかの悪さをしていることが想像できる。また今回の実験では、あくまでも安定性の強い最終産物 6-keto PGF1 α をみているのであって PGI2 から 6-keto PGF1 α の生成経路に何かがあることも考えられる。いずれにしろ我々は、温阻血再灌流障害時にはアラキドン酸カスケードの代謝経路が Prostaglandine 合成から Thromboxane 合成の方向へシフトしていると考えている。そして、この作用のため類洞の狭小化、類洞内皮細胞障害、類洞内凝固といった現象が起き、微小循環障害へと続いていくと考えられる。今まで温阻血再灌流障害とアラキドン酸カスケードの関連について述べた報告は極めて少ないが、これらの結果から、アラキドン酸カスケードはフォスフォリパーゼ A や Cox をその key enzyme としてもち、温阻血再灌流障害に強く関与していることが示唆された。

以上のように我々は、温阻血再灌流障害の大きな原因として、炎症性サイトカイン等による類洞内皮細胞障害や類洞内好中球集積による微小循環障害のみならず、アラキドン酸カスケードの活性化や細胞膜におけるアラキドン酸の増加が関与していると考えている。細胞膜におけるアラキドン酸の増加は、膜の脆弱性につながり、細胞構造の破壊を引き起こすことが知られている[45,46,49,50]。このため我々は、アラキドン酸の総量を推測する必要があると考え、TXB2、6-keto PGF1 α に加え、もう1つのアラキドン酸代謝物である Leukotriene B4 も測定することとした。その結果 NHB 群に対し NM・FR+NM 群で有意に Leukotriene B4 の低下が認められたことから、nafamostat mesilate のフォスフォリパーゼ A2 抑制作用によりアラキドン酸が減少している可能性が示唆された。これらの知見からもアラキドン酸カスケードの抑制は温阻血再灌流障害の軽減において重要だと考えられた。

結論として、我々は TNF-α、IL-1β及び phospholipase A2 の抑制によって転写因子やアラキドン酸カスケードの活性化を抑制することに成功した。そしてその結果、心停止ドナーからの肝グラフトにおける温阻血再灌流障害を軽減することに成功した。

7. 結語

心停止ドナーからの肝移植では、死戦期における IL-1 β ・TNF- α の抑制およびアラキドン酸カスケードの抑制が重要であると考えられた。FR167653 と nafamostat mesilate の2剤併用療法は、屍体肝グラフトのコンディショニング法として効果があると考えられた。

8. 参考文献

1. Harper AM. The OPTN Waiting List, 1988-1999. In: Cecka JM, Terasaki PI, eds. CLINICAL TRANSPLANTS 2000. Richmond, Virginia: United Network for Organ Sharing; 2000: 73-83.
2. Melemdz HV and Heaton ND. Understanding "marginal" liver grafts. Transplantation 1999; 68(4): 469-471.
3. Totsuka E, Fung JJ, Hakamada K, et al. Experience of orthotopic liver transplantation from non-heart-beating donors at the University of Pittsburgh Medical Center. Nippon Geka Gakkai Zasshi 1999; 100(12): 818-821.
4. Shibuya H, Ohkohchi N, Seya K, et al. Kupffer cells generate superoxide anions and modulate reperfusion injury in rat livers after cold preservation. Hepatology 1997; 25: 356-360.
5. Bremer C, Bradford BU, Hunt KJ, et al. Role of Kupffer cells in the pathogenesis of hepatic reperfusion injury. Am J Physiol 1994; 267: G630-636.
6. Caldwell-Kenkel JC, Currin RT, Tanaka Y, et al. Kupffer cells activation and endothelial cells damage after storage of rat livers: effect of reperfusion. Hepatology 1991; 13: 83-95.

7. Thurman RG, Lindert KA, Cowper KB, et al. Activation of Kupffer cells following liver transplantation. In Wisse E, Knook DL, McCuskey RS, eds. Cells of the Hepatic Sinusoid. Kupffer Cell Foundation, Linden 1991; 3: 358-363.

8. Ohkohchi N, Sakurada M, Endoh T, et al. Role of free radicals and energy synthesis on primary graft nonfunction in liver transplantion. Transplant Proc 1991; 23(5): 2416-2419.

9. Ohkohchi N, Endoh T, Oikawa K, et al. Fragility of the electron transport chain and superoxide generation in mitochondria of the liver graft after cold ischemia. Transplantation 1999; 67(8): 1173-1177.

10. Tsukamoto S, Ohkohchi N, Fukumori T, et al. Elimination of Kupffer cells and nafamostat mesilate rinse prevent reperfusion injury in liver grafts from agonal non-heart beating donors. Transplantation 1999; 67(11): 1396-1403.

11. Shibuya H, Ohkohchi N, Tsukamoto S, et al. Tumor necrosis factor-induced, superoxide-mediated neutrophil accumulation in cold ischemic /reperfused rat liver. Hepatology 1997; 26: 113-120.

12. Yamamoto N, Sakai F, Yamazaki H, et al. FR167653, a dual inhibitor of interleukin-1 and tumor necrosis factor-alpha, ameliorates endotoxin-induced shock. Eur J Pharmacol 1997; 327(2-3): 169-174.

１３． Oikawa K, Ohkohchi N, Sato M, et al. Kupffer cells play an important role in the cytokine production and activation of nuclear factors of liver grafts from non-heart-beating donors. Transpl Int 2002; 15: 397-405.

１４． Gores GJ, Kost LJ, and LaRusso NF. The isolated perfused rat liver: conceptual and practical considerations. Hepatology 1985; 6: 511-517.

１５． Boya P, Larrea E, Sola I, et al. Nuclear factor-kappa B in the liver of patients with chronic hepatitis C: decreased RelA expression is associated with enhanced fibrosis progression. Hepatology 2001; 34(5): 1041-1048.

１６． Dignam J D, Lebovitz RM and Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 1983; 11(5): 1475-1489.

１７． O'Neill GP and Ford-Hutchison AW. Expression of mRNA for cyclooxygenase-1 and cyclooxgenase-2 in human tissues. FEBS Lett. 1993; 330(2): 156-60.

１８． Koizumi M, Ohkohchi N, Katoh H, et al. Preservation and reflow damage in liver transplantation in the pig. Transplant Proc 1989; 21: 1323-1326.

１９． Malago M, Hertl M, Testa G, et al. Split-liver Transplantation: Future use of scarce donor organs. World J Surg 2002; 26(2): 275-282.

２０． D'Alessandro AM, Hoffmann RM, Knechtle SJ, et al. Liver transplantation from

controlled non-heart-beating donors. Surgery 2000; 128(4): 579-588.

２１. Casavilla A, Ramirez C, Shapiro R, et al. Experience with liver and kidney allografts from non-heart-beating donors. Transplantation 1995; 59(2): 197-203.

２２. Lutterova M, Szatmary Z, Kukan M, et al. Marked difference in tumor necrosis factor-alpha expression in warm ischemia- and cold ischemia-reperfusion of the rat liver. Cryobiology 2000; 41(4): 301-314.

２３. Kukan M, Bezek S, and Trnovec T. Role of hepatovasculature in warm ischaemia-reperfusion injury of rat liver. Physiol Res 1996; 45(5): 427-430.

２４. Oikawa K, Ohkohchi N, Sato M, et al. Elimination of Kupffer cells suppresses activation of nuclear factor kappa B and production of cytokines and eicosanoids in non-heart-beating donors. Transplantat Proc 2001; 33(1-2): 839-840.

２５. Hashimoto K, Nishizaki T, Yoshizumi T, et al. Beneficial effect of FR167653 on cold ischemia/reperfusion injury in rat liver transplantation. Transplantation 2000; 70(9): 1318-22.

２６. Caty MG, Guice KS, Oldham KT, et al. Evidence for tumor necrosis factor-induced pulmonary microvascular injury after intestinal ischemia-reperfusion injury. Ann Surg 1990; 212(6): 694-700.

２７. Simpson R, Alon R, Kobzik L, et al. Neutrophil and nonneutrophil-mediated injury in

intestinal ischemia-reperfusion. Ann Surg 1993; 218(4): 444-453.

２８． Koike K, Yamamoto Y, Hori Y, et al. Group IIA phospholipase A2 mediates lung injury in intestinal ischemia-reperfusion. Ann Surg 2000; 232(1): 90-97.

２９． Zhang Y, Ohkohchi N, Oikawa K, et al. Assessment of viability of the liver graft in different cardiac arrest models. Transplant Proc 2000; 32: 2345-2347.

３０． Malinin NL, Boldin MP, Kovalenko AV, et al. MAP3K-related kinase involved in NF-kappa B induction by TNF, CD-95, and IL-1. Nature 1997; 385: 540-544.

３１． Bradham CA, Schemmer P, Stachlewitz RF, et al. Activation of nuclear factor-kappa B during orthotopic liver transplantation in rats is protective and does not require Kupffer cells. Liver Transpl Surg 1999; 5(4): 282-293.

３２． Mayes PA. Metabolism of unsaturated fatty acid and eicosanoids. In: Murray RK, Granner DK, Mayes PA, and Rodwell VW, eds. Harper's Biochemistry 25th Edition. Stanford, CT: Appleton & Lange; 2000: 250-258.

３３． Voet D and Voet JG. Enzymatic catalysis. In: Voet D, Voet JG, eds. Biochemistry 2nd Edition. New York: John Wiley & Sons, Inc.; 1995: 371-410.

３４． Gong C, Ennis SR, Hoff JT, et al. Inducible cyclooxgenase-2 expression after experimental intercerebral hemorrhage. Brain Res 2001; 901(1-2): 38-46

３５． Shornick LP, De Togni P, Mariathasan S, et al. Mice deficient in IL-1beta manifest

impaired contact hypersensitivity to trinitrochlorobenzone. J Exp Med. 1996; 183(4): 1427-1436.

３６. Grandel U, Fink L, Blum A, et al. Endotoxin-induced myocardial tumor necrosis factor-α synthesis depresses contractility of isolated rat hearts. Circulation 2000; 102(22): 2758.

３７. Nanji AA, Jokelainen K, Fotouhinia A, et al. Increased severity of alcoholic liver injury in female rats: role of oxidative stress, endotoxin, and chemokines. Am J Physiol Gastrointest Liver Physiol 2001; 281(6): G1348-1356.

３８. Nanji AA, Jokelainen K, Tipoe GL, et al. Dietary saturated fatty acids reverse inflammatory and fibrotic changes in rat liver despite continued ethanol administration. J Pharmacol Exp Ther 2001; 299(2): 638-644.

３９. Fiebich BL, Mueksch B, Boehringer M, et al. Interleukin-1beta induces cyclooxgenase-2 and prostaglandin E(2) synthesis in human neuroblastoma cells: involvement of p38 mitogen-activated protein kinase and nuclear factor-kappa B. J Neurochem 2000; 75(5): 2020-2028.

４０. Lim JW, Kim H, and Kim KH. Nuclear factor-kappa B regulates cyclooxgenase-2 expression and cell proliferation in human gastric cancer cells. Lab Invest 2001; 81(3): 349-360.

41. Okamoto T and Hino O. Expression of cyclooxgenase-1 and −2 mRNA in rat tissues: tissue-specific difference in the expression of the basal level of mRNA. Int J Mol Med 2000; 6(4): 455-457.

42. Nakamura H, Nemenoff RA, Gronich JH, and Bonventre JV. Subcellular characteristics of phospholipase A2 enzymatic activity after renal ischemia and reperfusion. J Clin Invest 1991; 87(5): 1810-1818.

43. Fukuzawa K, Emre S, Senyuz O, et al. N-acetylcysteine ameliorates reperfusion injury after warm hepatic ischemia. Transplantation 1995; 59: 6-9.

44. Marubayashi S, Takenaka M, Dohi K, et al. Adenine nucleotide metabolism during hepatic ischemia and subsequent blood reflow period and its relation to organ viability. Transplantation 1980; 30: 294-296.

45. Gasbarrini A, Borle AB, Farghali H, et al. Effect of anoxia on intracellular ATP, Na^{+}, Ca^{2+}, Mg^{2+} and cytotoxicity in rat hepatocytes. J Biol Chem 1992; 267: 6654-6663.

46. Inoue T, Yoshida Y, Nishimura M, et al. Ca^{2+}-induced, phospholipase-independent injury duaring reoxygenation of anoxic mitochondria. Biochem Biophys Acta. 1993; 1140: 313-320.

47. Yokoyama I, Kobayashi T, Negita M, et al. Liberation of vasoactive substances and its prevention with thromboxane A2 synthase inhibitor in pig liver transplantation.

Transpl Int 1996; 9(1): 76-81.

４８. Yokoyama I, Negita M, Kobayashi T, et al. Beneficial effect of donor pretreatment with thromboxane A2 synthase inhibitor on the graft survival in pig liver transplantation. J Surg Res 1996; 60(1): 232-238.

４９. Hopkins GJ, and West CE. Diet-induced changes in the fatty acid composition of mouse hepatocyte plasma membranes. Lipids 1977; 12(4): 327-334.

５０. Horton AA, and Wood JM. Prevention of thromboxane B2-induced hepatocyte plasma membrane bleb formation by certain prostaglandins and a protease inhibitor. Biochim Biophys Acta 1990; 1022(3): 319-324.

9. 図・表

Groups	The agonal time until cardiac arrest. (minutes)
NHB	9.232±2.153
NM	9.500±3.082
FR	9.066±2.046
FR+NM	9.398±1.845

Table 1: The agonal time between apnea to cardiac arrest.

Values represent the mean ±SD.

Groups	Portal venous flow (ml/kg/h)	Bile production (μl/kg/h)	AST (Karmen units)	LDH (Wrobleski units)
HB	3145±651*	794±188*	0.8±0.9*	8.1±6.9*
NHB	1889±612	419±176	25.5±13.4	83.0±70.6
NM	3443±449*	600±158	1.5±1.2*	21.8±28.0
FR	3032±306*	699±157*	0.8±0.7*	4.7±3.0*
FR+NM	3803±146**†	836±109**‡	1.0±0.6*	7.1±1.9*

Table 2: Portal flow volume, bile production, AST and LDH measured 1 hr after reperfusion.

Values represent the mean ±SD. AST, aspartate aminotransferase; LDH, lactate dehydrogenase; HB, heart-beating group; NHB, non-heart-beating group; NM, nafamostat mesilate group; FR, FR167653 group; FR+NM, FR167653 + nafamostat mesilate group. * P<0.05 vs. NHB group; ** P<0.005 vs. NHB group; † P<0.05 vs. FR group; ‡ P<0.05 vs. NM group.

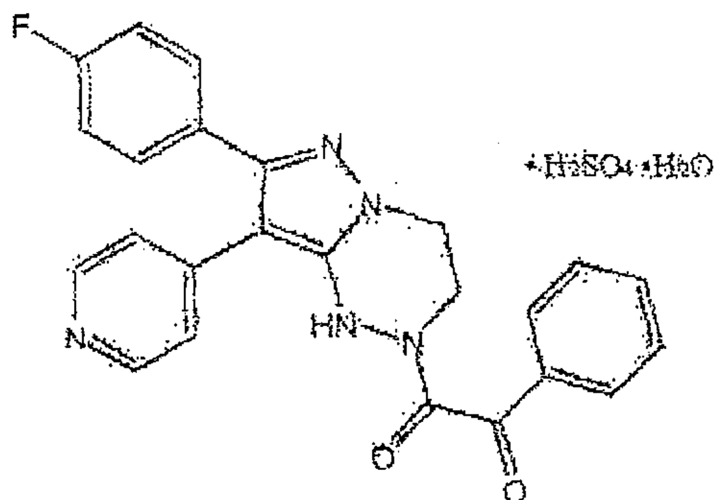


Figure 1: Chemical structure of FR167653.

1[7-(4-fluorophenyl) 1,2,3,4-tetrahydro-8-(4-pyridyl) pyrazolo[5,1-c][1,2,4] triazin-2-yl]-2-phenylethanedione sulfate monohydrate.

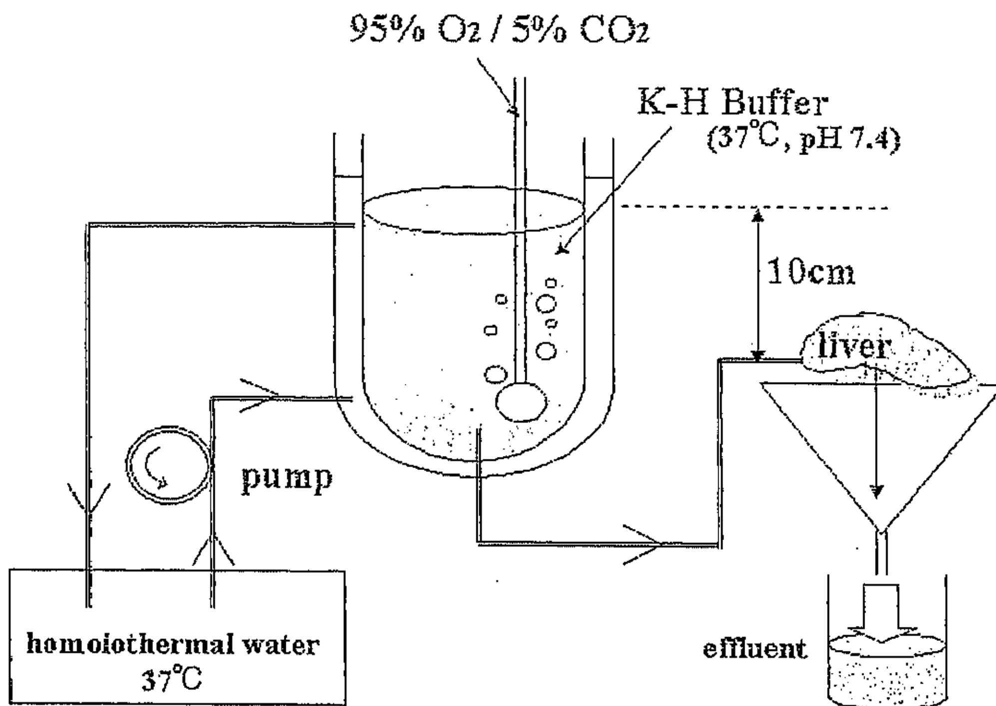
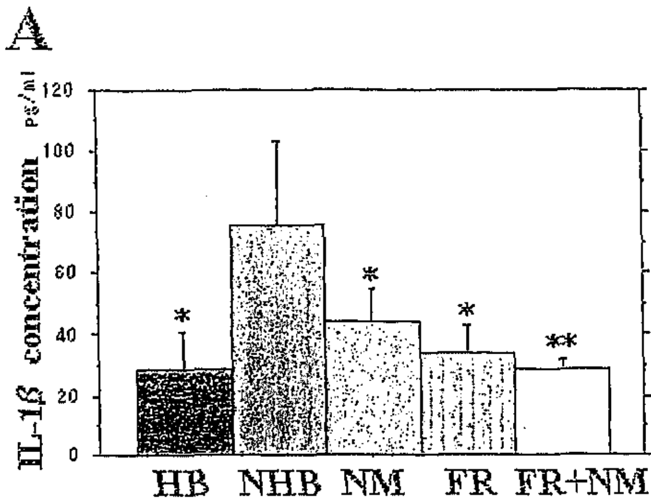


Figure 2: Perfusion system to collect the perfusate.

K-H Buffer, Krebs-Henseleit bicarbonate buffer.



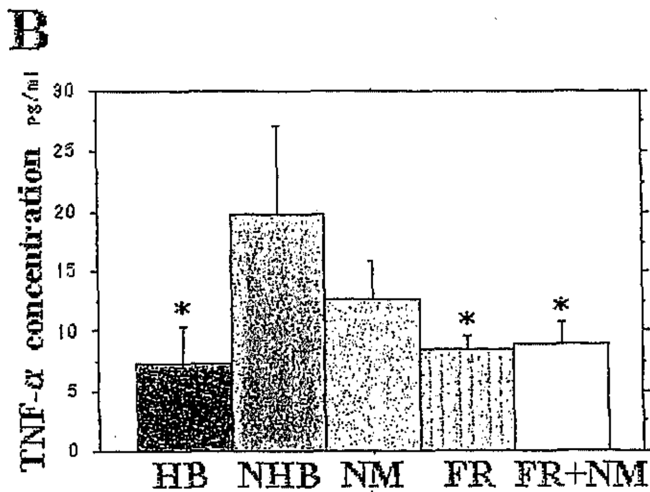


Figure 3: Concentration of inflammatory cytokines (IL1-β (A), TNF-α (B)) in the perfusate.

HB, heart-beating group; NHB, non-heart-beating group; NM, nafamostat mesilate group; FR, FR167653 group; FR+NM, FR167653 + nafamostat mesilate group. All livers were perfused for 60 minutes with Krebs-Henseleit bicarbonate buffer after 6 hours cold preservation. * $P<0.05$ vs. NHB group; ** $P<0.005$ vs. NHB group. Error bar= ±SD.

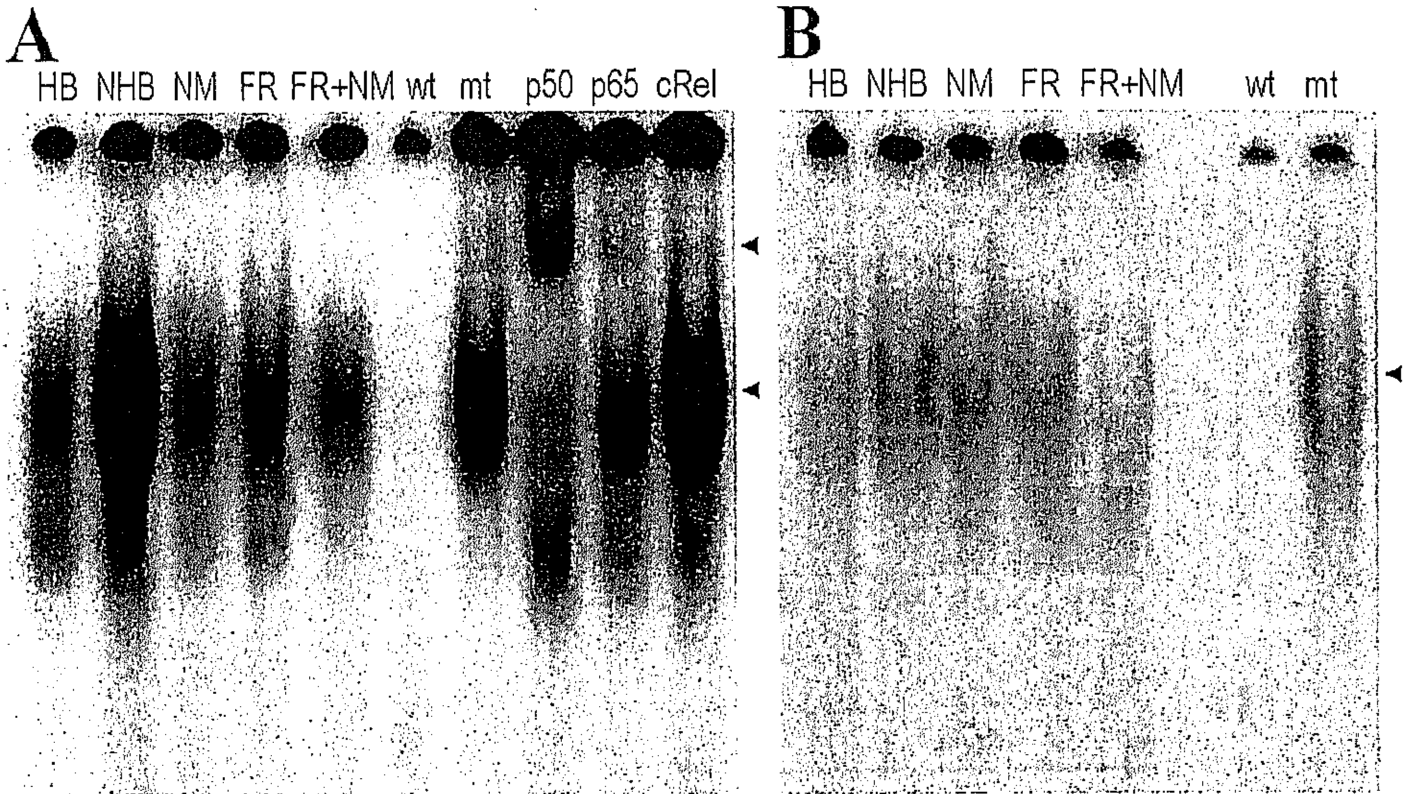


Figure 4: Activated signals of transcription factors [NF-κB (A) and AP1 (B)] in liver tissues after reperfusion.

HB, heart-beating group; NHB, non-heart-beating group; NM, nafamostat mesilate group; FR, FR167653 group; FR+NM, FR167653 + nafamostat mesilate group; wt, competition assay with wild type; mt, competition assay with mutant type; p50, super shift assay with p50 antibody; p65, super shift assay with p65 antibody; cRel, super shift assay with cRel antibody.

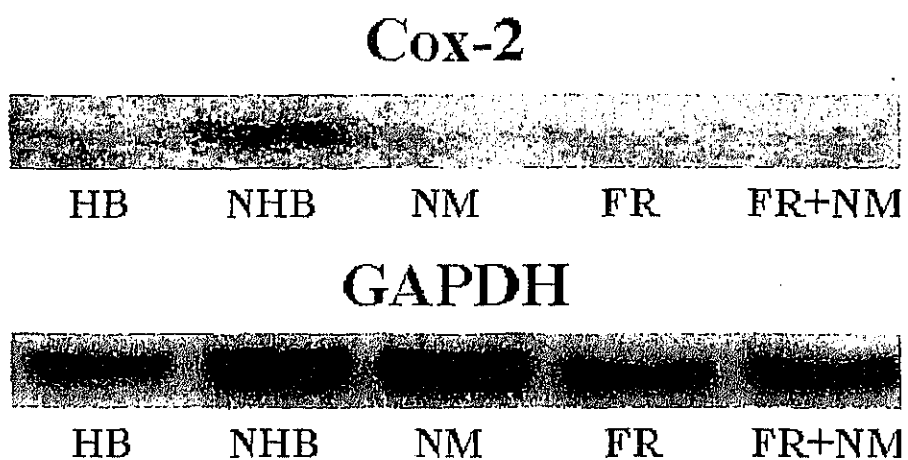
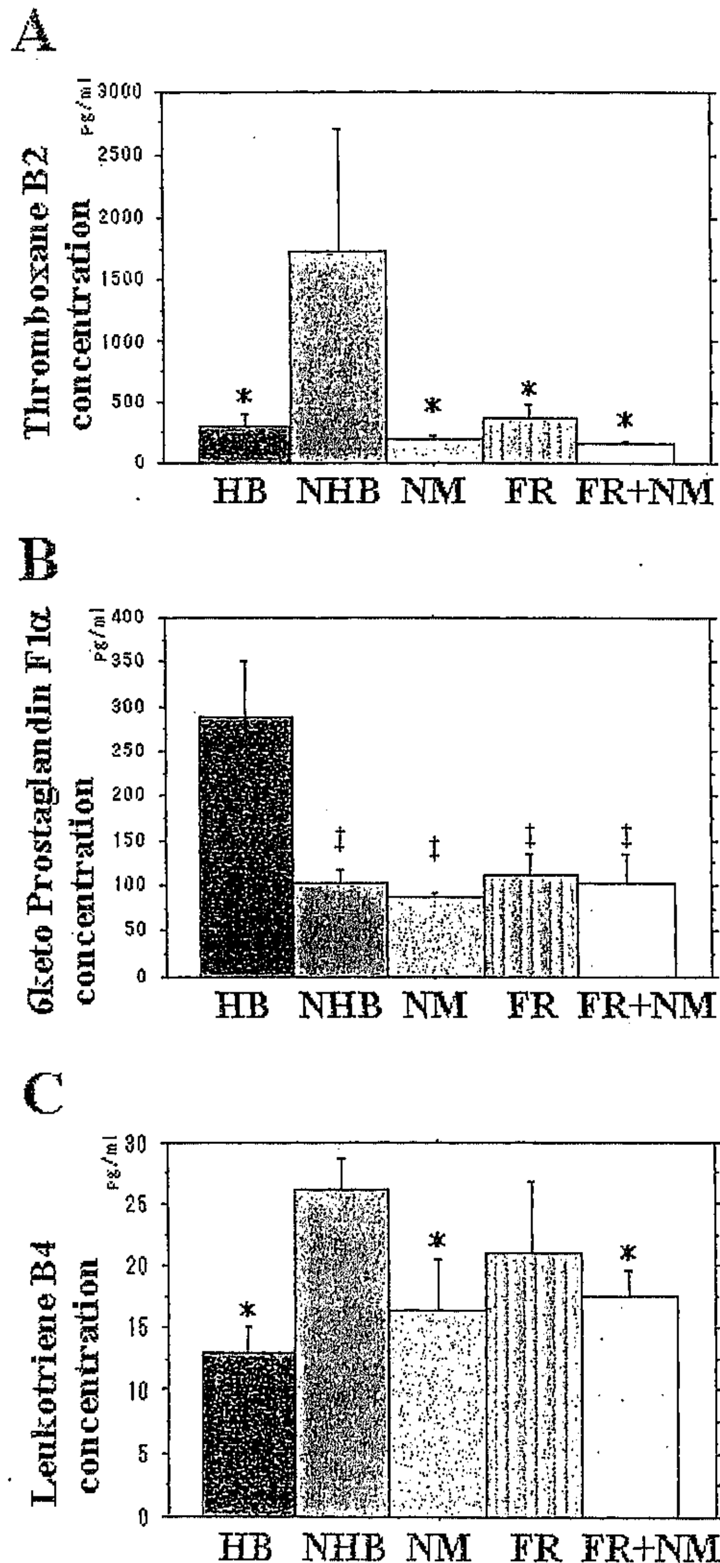


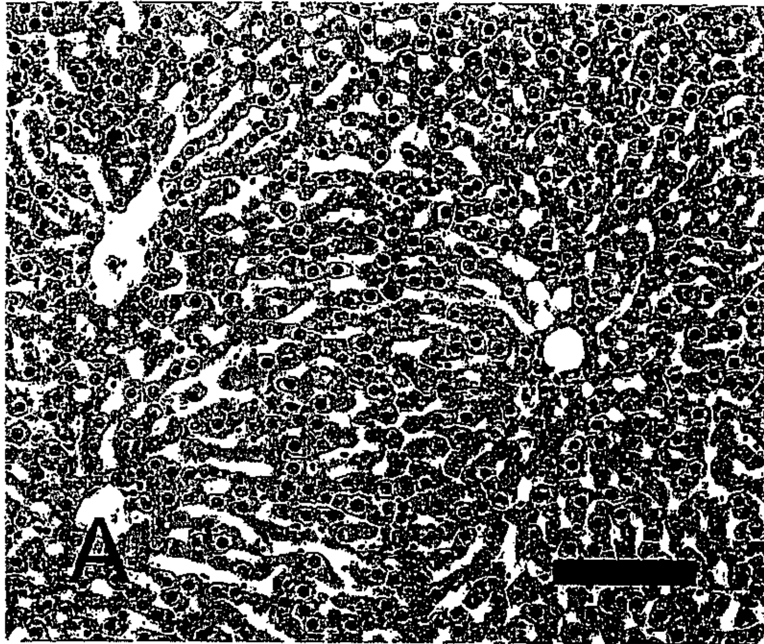
Figure 5: Northern blot analysis of cyclooxygenase-2 (Cox-2) mRNA in the liver tissue after reperfusion.

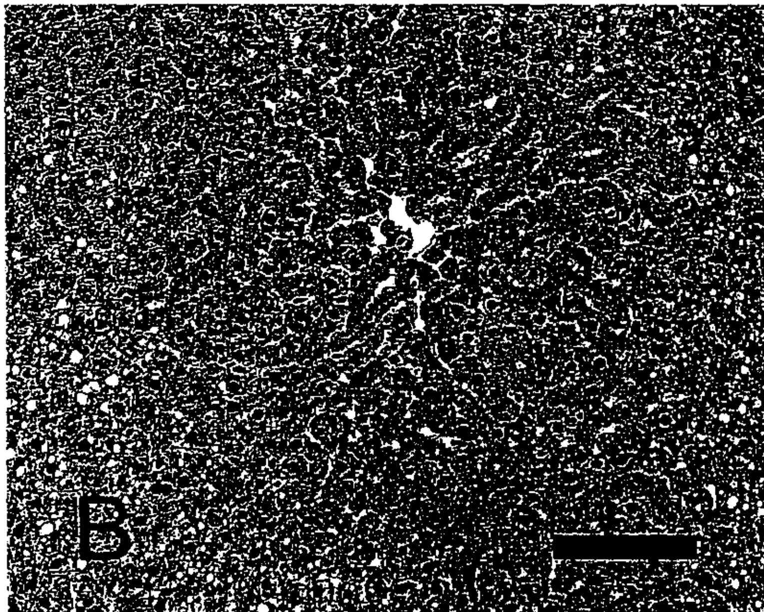
GAPDH served as the internal control. HB, heart-beating group; NHB, non-heart-beating group; NM, nafamostat mesilate group; FR, FR167653 group; FR+NM, FR167653 + nafamostat mesilate group.

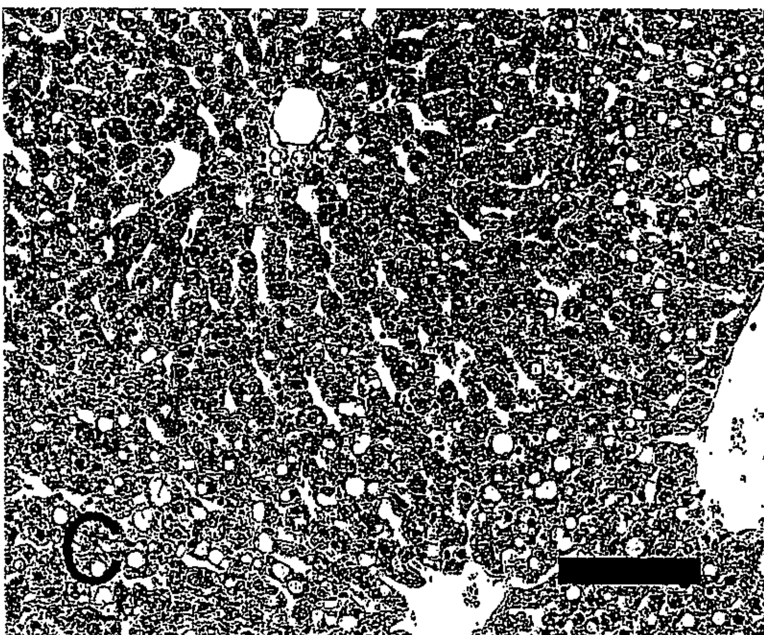
Figure 6:
Concentration of the products of the arachidonic acid cascade in the perfusate.

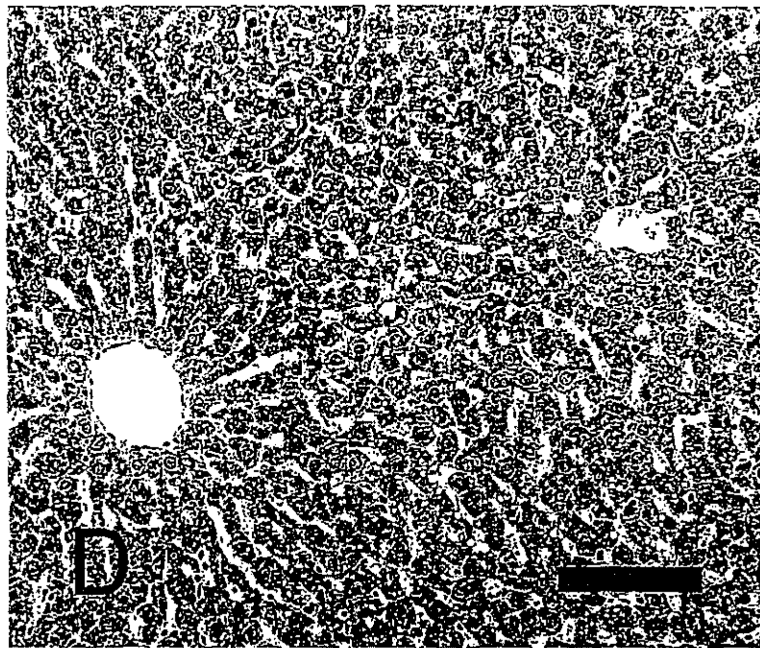
HB, heart-beating group; NHB, non-heart-beating group; NM, nafamostat mesilate group; FR, FR167653 group; FR+NM, FR167653 + nafamostat mesilate group.
*$P<0.05$ vs. NHB group;
‡ $P<0.005$ vs. HB group.
Error bar= ±SD.




A


B


C



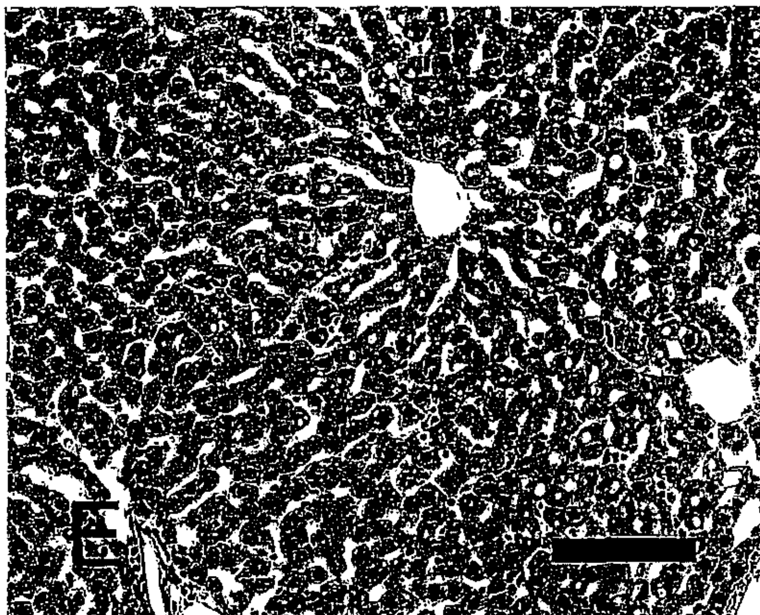
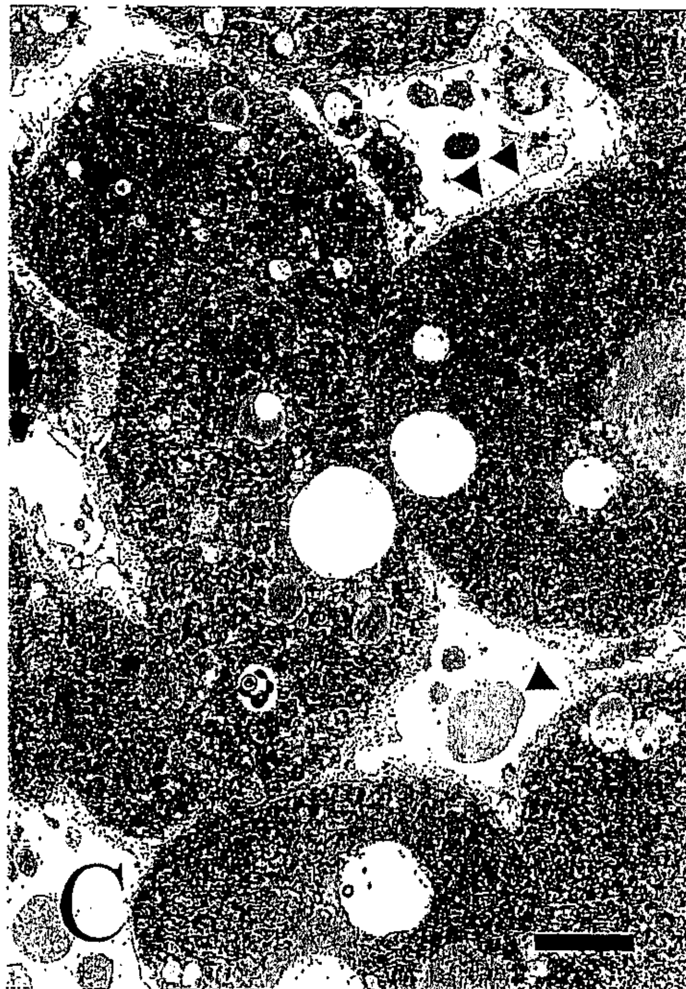


Figure 7: Light microphotographs of the liver grafts from the HB (A), NHB (B), NM (C), FR (D), and FR+NM (E) groups, after reperfusion.

(A) The structure of sinusoids was well preserved. (B) The structure of sinusoids was completely destroyed, and sinusoidal lumina could not be identified. (C), (D) There was only a slight destruction of sinusoids but the endoplasmic reticulum showed vesiculation in some areas. (E) There was no remarkable destruction of sinusoids and the lumen of sinusoids was wide. Scale bar=100 μm.

A

B

C

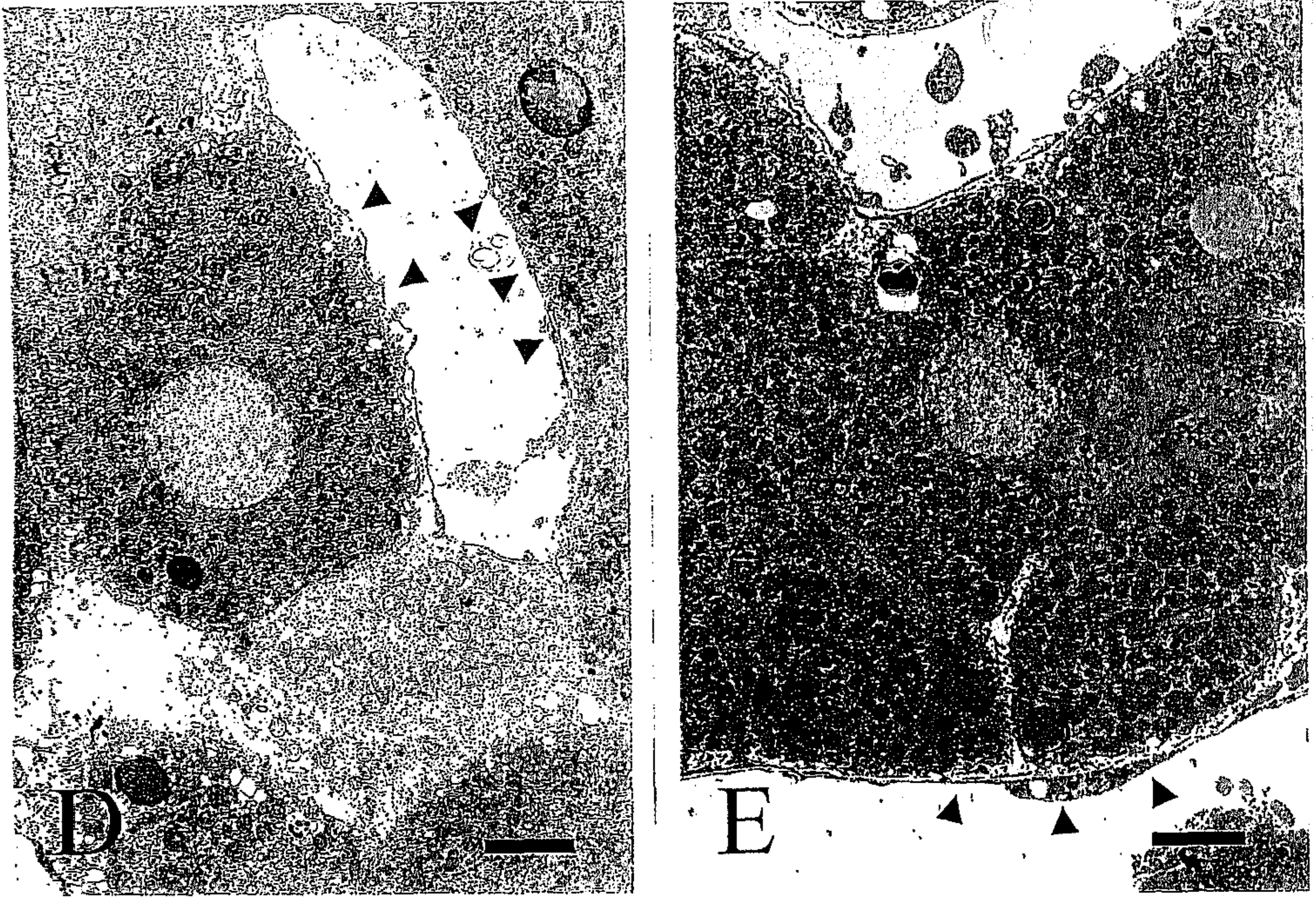


Figure 8: Transmission electron microphotographs of the liver grafts from the HB (A), NHB (B), NM (C), FR (D), and FR+NM (E) groups, after reperfusion.

(A) Most sinusoidal endothelial cells were well preserved, with the Disse's spaces intact (arrowheads), the structure of sinusoids was also well preserved. (B) Sinusoidal endothelial cells were completely destroyed. Hepatocytes swelled with sparsing cytoplasm into sinusoidal space (arrowheads). (C) Hepatocytes were not swollen and the lumen of sinusoids was wide. As a characteristic finding of this group, enlarged Disse's spaces and hepatocytes with few microvilli were observed in many areas, the sinusoidal lining of endothelial cells showed discontinuity in many parts (arrowheads), and the endoplasmic reticulum showed vesiculation. (D) Most sinusoidal endothelial cells were preserved but in some parts the sinusoidal lining showed discontinuity (arrowheads). (E) Most sinusoidal endothelial cells were well preserved the same as those in the HB group and Disse's spaces also were intact (arrowheads). In addition, hepatocytes were not swollen and the lumen of sinusoids was wide. Scale bar=5 μm.

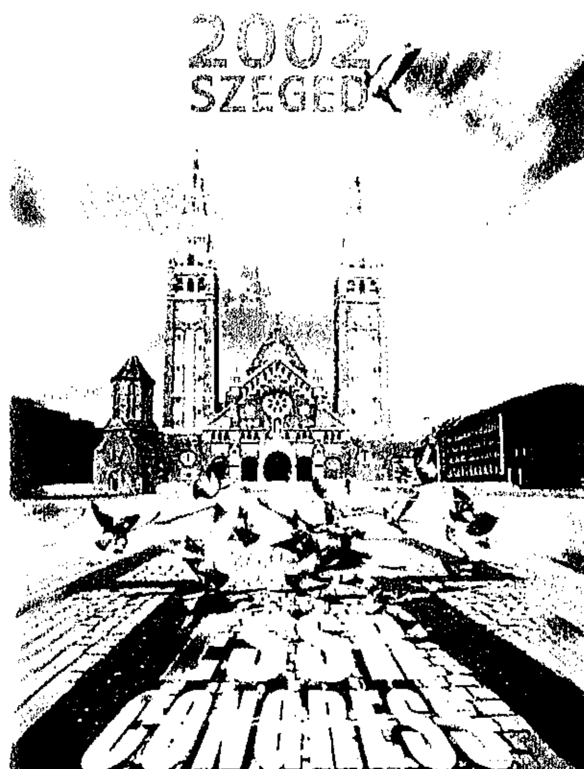
Reprinted from:

Proceedings of the 37th Congress of the

EUROPEAN SOCIETY FOR SURGICAL RESEARCH

Szeged (Hungary), May 23-25, 2002

Editor
MIHALY BOROS



MONDUZZI EDITORE
INTERNATIONAL PROCEEDINGS DIVISION

The Effects of FR167653 On Warm Ischemia/Reperfusion Injury of the Liver Grafts from NHBD

S. Miyagi, N. Ohkohchi, K. Oikawa, M. Satoh, S. Tsukamoto and S. Satomi

Division of Advanced Surgical Science and Technology
Tohoku University, Sendai, Japan

Summary

The purpose is to investigate the cytoprotective effects of FR167653 and protease inhibitor on warm ischemia/reperfusion injury and establish the procurement of the graft for successful liver transplantation from NHBDs. Male Wistar rats were divided into four groups as follows (n=5): **HB group**, livers were retrieved from heart beating donors; **NHB group**, from NHBDs; **FR group**, from NHBDs pretreated with FR167653 (2mg/kg); **FRnm group**, from NHBDs pretreated with FR167653 and rinsed with protease inhibitor. These livers were perfused for 60 minutes after cold preservation. The result was that FR167653 and protease inhibitor reduced inflammatory cytokines, mediator of arachidonic acid cascade, and the injury of microcirculatory in the liver grafts from NHBDs.

Introduction

Liver transplantation has become a standard therapy for end-stage liver diseases. But shortage of donor became big problem all over the world[1]. Recently some institutes tried to carry out transplantation from non-heart-beating donors (NHBDs) clinically. However, the criteria and the retrieval procedure of the grafts from NHBDs are not established yet[2,3]. The purpose of this study is to investigate the cytoprotective effects of FR167653, which is suppressant of the release of TNF-α and IL-1β[4], and nafamostat mesilate, which contains a strong protease inhibitor, on warm ischemia/reperfusion injury, and to establish the procurement of the grafts for successful liver transplantation from NHBDs.

©2002 by Monduzzi Editore S.p.A. – MEDIMOND Inc. C523C0151

*Table 1: Values of portal flow volume, bile production, AST, and LDH, after reperfusion. * P<0.05 vs. NHB group.*

Groups	Portal venous flow (ml/kg/h)	Bile production (μl/kg/h)	AST (Karmen units)	LDH (Wrobleski units)
HB	3145±651*	794±188*	0.8±0.9*	8.1±6.9*
NHB	1889±612	419±176	25.5±13.4	83.0±70.6
FR	3032±306*	699±157*	0.8±0.7*	4.7±3.0*
FRnm	2959±286*	792±119*	0.4±0.8*	1.8±2.4*

*P<0.05 vs NHB

Materials and Methods

Male Wistar rats were used. Livers with/without agonal condition were retrieved and perfused for 60 minutes with Krebs-Henseleit bicarbonate buffer[5] after 6 hours cold preservation. Animals were divided into four groups (n=5): **HB group**, livers were isolated from heart beating donors; **NHB group**, livers were isolated from NHBDs; **FR group**, livers were isolated from NHBDs pretreated with FR167653 (2mg/kg); **FRnm group**, livers were isolated from NHBDs pretreated with FR167653 and were rinsed with nafamostat mesilate, which contained a strong serine protease inhibitor. **Examinatiuon items:** Bile production and portal flow were measured. IL-1β, TNF-α, and prod-

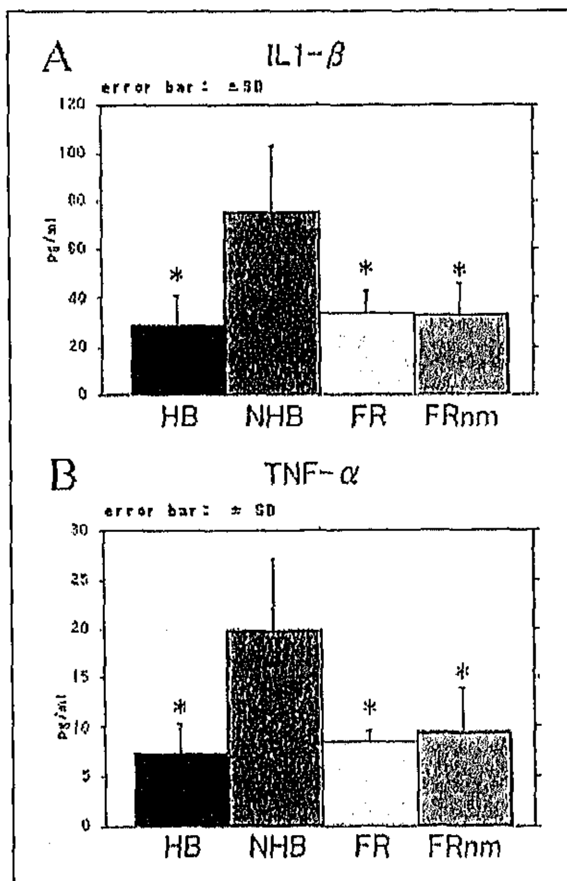


*Figure 1: Concentration of inflammatory cytokines (IL1-β (A), TNF-α (B)) in perfusate. * P<0.05 vs. NHB group.*

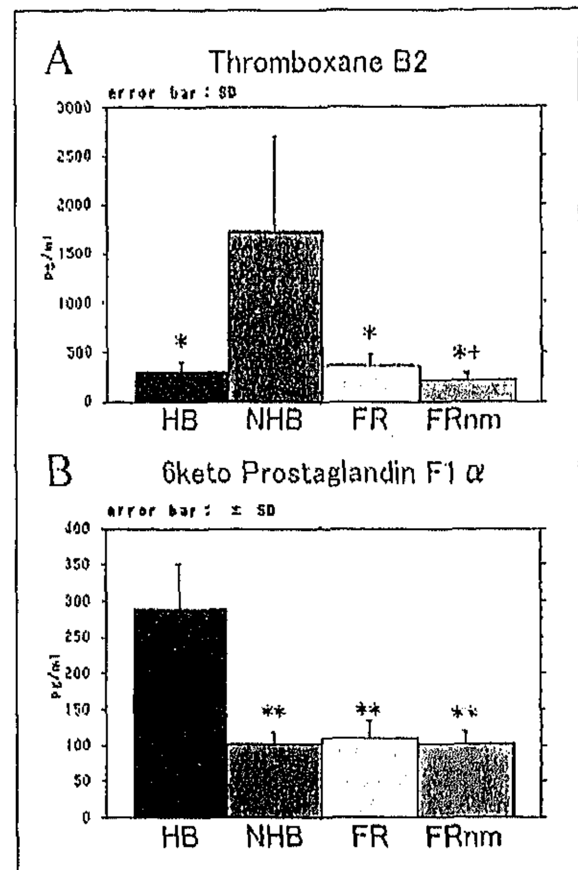


*Figure 2: Concentration of the products of arachidonic acid cascade (thromboxane B2 (A), 6-keto prostaglandin F1α (B)) in perfusate. * $P<0.05$ vs. NHB group; + $P<0.05$ vs. FR group; ** $P<0.005$ vs. HB group.*

ucts of arachidonic acid cascade (thromboxane B2, 6-keto prostaglandin F1α) were measured with a commercially available enzyme-linked immunosorbent assay kits (Cayman Chemical, Ann Arbor, MI, USA). Transcription factors (NF-κB, AP1, STAT3) were measured by the electrophoretic mobility shift assay[6]. Histological findings were also investigated.

Results

Flow volume and bile production of NHB group decreased significantly compared with those in HB group, but those of FR and FRnm groups were maintained as the same level of HB group (Table 1). In NHB group, values of IL-1β, TNF-α, and thromboxane B2, expressions of NF-κB, AP1, and STAT3 were significantly higher but those values of FR and FRnm groups were low the same as in HB group (Figure 1, 2, 3). In NHB group sinusoidal endothelial cells were completely destroyed, hepatocytes were swollen. On the other hand, in both FR and FRnm groups the structure of sinusoids was well preserved and sinusoidal lumen was maintained wide as same as those in HB group (Figure 4).

Conclusion

The new drug, FR167653 remarkably inhibits the release of IL-1β and

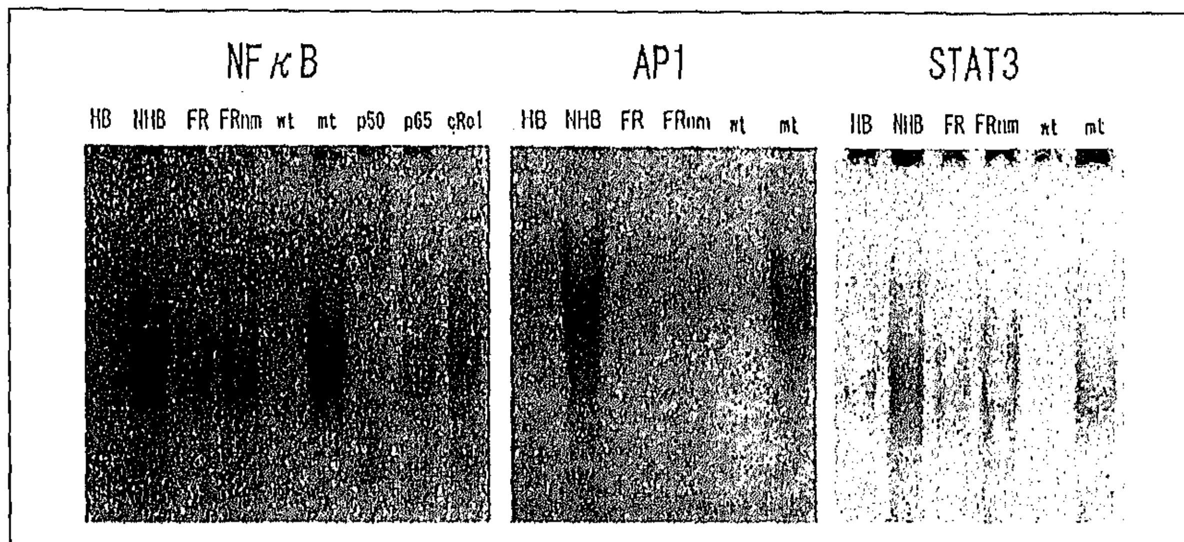


Figure 3: The activated signals of transcription factor (NF-κB, AP1, STAT3) in liver tissues. wt, competition assay with wild type; mt, competition assay with mutant type.

TNF-α of the liver at reperfusion after agonal warm ischemia, and the suppression of those cytokines and the administration of protease inhibitor were strongly associated with maintenance of sinusoidal microcirculatory structure in the liver graft from NHBD.

Figure 4: Transmission electron microphotographs of the liver grafts from HB group (A), NHB group (B), FR group (C), and FRnm group (D), after reperfusion. (A) Most of sinusoidal endothelial cells were well maintained, with the intact Disse's space, the structure of sinusoids was well preserved. (B) Sinusoidal endothelial cells were completely destroyed, and microvilli of hepatocytes were poured to the sinusoidal lumen. Hepatocytes swoll with sparsing cytoplasm into sinusoidal space. (C) Most of sinusoidal endothelial cells were well maintained but in some part the sinusoidal lining lost its continuity. (D) Most of sinusoidal endothelial cells were well maintained same as those in HB group and Disse's space was also intact. In addition hepatocytes did not swell and the space of sinusoidal lumen was maintained wide. Scale bar=5 μm.

Reference

1. Harper AM. The OPTN Waiting List, 1988-1999. In: Cecka JM, Terasaki PI, eds. CLINICAL TRANSPLANTS 2000. Richmond, Virginia: United Network for Organ Sharing: 73-83: 2000.
2. Melemdz HV. and Heaton ND. Understanding "marginal" liver grafts. Transplantation; 68(4): 469-471: 1999.
3. Totsuka E, Fung JJ, Hakamada K, Narumi S, and Sasaki M. Experience of orthotopic liver transplantation from non-heart-beating donors at the University of Pittsburgh Medical Center. Nippon Geka Gakkai Zasshi; 100(12): 818-821: 1999.
4. Yamamoto N, Sakai F, Yamazaki H, Nakahara K, and Okuhara M. FR167653, a dual inhibitor of interleukin-1 and tumor necrosis factor-alpha, ameliorates endotoxin-induced shock. Eur. J. Pharmacology; 327(2-3):169-174: 1997.
5. Gores GJ, Kost LJ, and LaRusso NF. The isolated perfused rat liver:conceptual and practical considerations. Hepatology; 6: 511-517: 1985.
6. Boya P, Larrea E, Sola I, Majano PL, Jimenez C, Civeira MP, and Prieto J. Nuclear factor-kappa B in the liver of patients with chronic hepatitis C: decreased RelA expression is associated with enhanced fibrosis progression. Hepatology; 34(5): 1041-1048: 2001.

課題４：臨床応用を前提とした小動物を用いた死戦期を伴う心臓死

ドナー肝の障害に対する薬剤の効果の検討

ラットを用いた移植実験

【方法】

1. 実験動物

２５０－３００ｇの雄性 Wistar ラット（日本チャールズ・リバー）を用い、自由給水給餌下に以下の各群に分け Kamada 法による肝移植実験を行った。

(1) Control 群：開胸により呼吸停止させ心停止を誘導、これを死戦期とし、心停止後 30 分の温阻血時間をおいた後肝摘出、移植した。ドナー、レシピエントともに手術開始時より 0.05ml/min で生理食塩水の輸液を行った。

(2) FR 群：Control 群と同様の手術を行い、同量の輸液内に FR167653（藤沢製薬）1 mg/kg/hr を投与した。

(3) PG 群：輸液内にプロスタグランジン I2 アナログ（OP2507, 小野薬品）33 ng/kg/min を投与した。

(4) NM 群：同様に nafamostat mesilate (フサン,鳥居薬品) 0.2 mg/kg/hr を投与した。

(5) FR+PG 群：FR 及び PG を同様に投与。

(6) FR+NM 群：FR 及び NM を投与。

(7) PG+NM 群：PG 及び NM を投与。

(8) FR+PG+NM 群：FR、PG 及び NM を投与。

各群の輸液量は同量とした。

2. ドナー手術、死戦期を伴うラット心停止モデル

ネンブタール（50mg/kg 腹腔内投与）により全身麻酔後、陰茎背静脈にシリコンチューブを挿入し輸液開始。正中切開に横切開を加え開腹した。ヘパリン（１ml/kg）を投与した。肝下部下大静脈及び門脈の分枝を結紮処理し肝臓を周囲より剥離。総胆管は長さ５mm のシリコンチューブを挿入固定後切離した。

dehydrogenase(LDH)を測定した(n=5)。

(3)組織学的検討

各群各犠牲死時に肝組織を採取し、10%中性ホルマリンで固定、ヘマトキシリン－エオジン染色にて光顕標本を作製した。

5．統計学的検討

データは mean±SD で表した。検定は一元配置分散分析及び多重比較検定（Tukey の方法）にて行い、生存率は Logrank 検定を行い、p＜0.05 を有意差ありとした。

【結果】

1. 生存率

7 日間の生存は Control 群(0/8)、FR 群(3/8)、PG 群(1/8)、NM 群(0/8)、FR+PG(6/8)群、FR+NM 群(2/7)、PG+NM(1/7)群、FR+PG+NM 群(5/8)、となり、FR+PG+NM 群と FR+PG 群(p<0.01)、及び FR 群(p<0.05)で有意に生存率が高かった（図１）。

2. 肝逸脱酵素

AST では各群間に有意差を認めなかった。図２参照。

ALT は、FR+PG+NM 群では 3 時間、24 時間ともに、FR+PG 群では 24 時間での値が Control 群より有意に低値であった(p<0.05)。図 3 参照。

LDH は、FR+PG+NM 群で 3 時間、24 時間ともに Control 群より低値であった(p<0.05)。図 4 参照。

3．組織学的検討

Control 群では類洞構造の破壊が著明で、肝細胞の空胞変性が広範に認められ

るが、生存率の改善を得た各群ではそれらの変化がやや軽微である。

【考察】

死戦期を伴うラット心停止肝移植モデルを用いて実験を行った。このモデルでは、肝グラフトは温阻血に加え、死戦期の低酸素や低血圧、腸管鬱血によるサイトカインの影響を受けると考えられる。Control 群ではその全例が生存しなかった。死亡例では腹水貯留が明らかで、肝不全による死と推定された。FR、PG、NM、の各薬剤を組み合わせて死戦期より投与開始することで、いくつかの群では生存率の改善を得ることができた。単独で改善があったのは FR のみで、PG、NM は単独ではその効果は明らかでなかった。しかし PG は FR とともに投与することで、それぞれを単独で用いるよりも有意に改善を認めた。また NM は、FR+PG+NM 群と FR+PG 群との間に生存率や肝酵素値の差は認めなかったが、前者の方が Control 群との間に有意差を示す検査値が多いことで、その効果が示唆される。しかし血中サイトカイン値や組織検査など、より詳細な検討が求められると思われる。

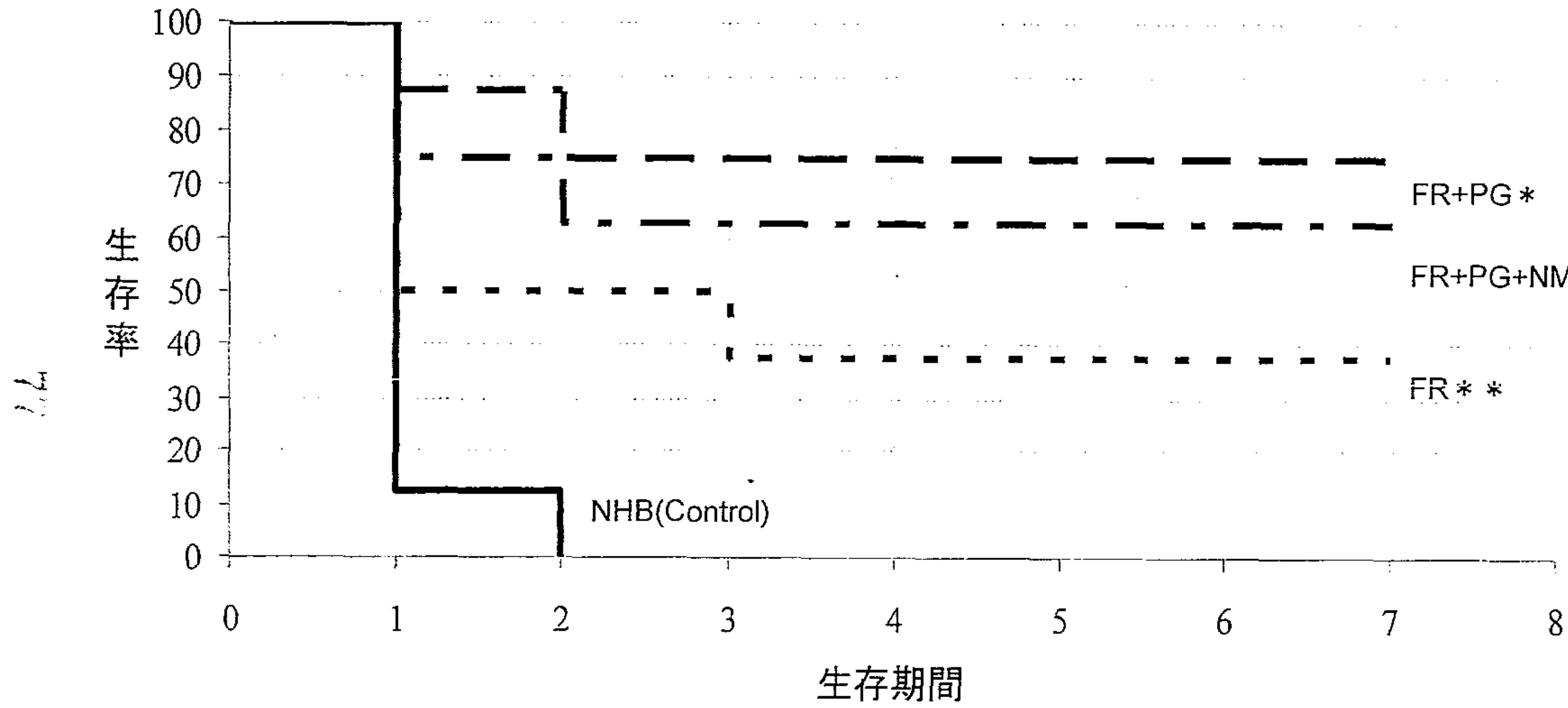
FR(IL-1β、TNF-α産生阻害剤)と PG(prostaglandinI2)を死戦期前より投与することで移植成績が改善した。死戦期においては、それらサイトカインの抑制と、エイコサノイドの産生をプロスタグランジン優位にすることが重要と推測される。NM(セリンプロテアーゼインヒビター)の効果については、なお検討の余地がある。

【まとめ】

1. 死戦期を伴う心停止ラット肝移植モデルは全例が生存しなかったが、FR、または FR と PG を投与することで改善した。

2． NM の効果など、より詳細な検討のため、血中サイトカインの変化や免疫染色、電子顕微鏡による組織の観察など、現在実験を進めている。

図1　生存曲線



＊　p＜0．01　　　＊＊　p＜0．05（vs　Control)

図2　血清AST

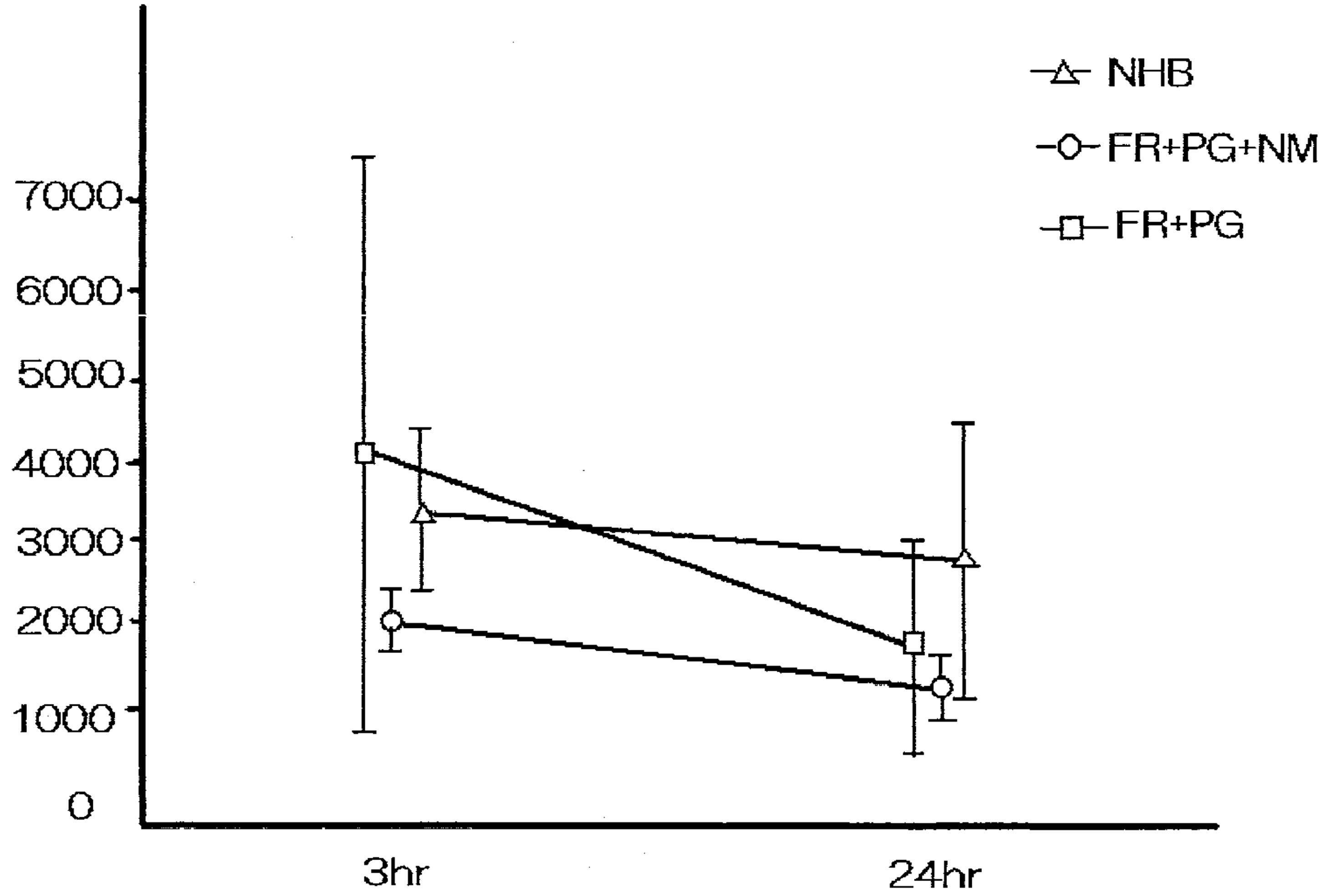
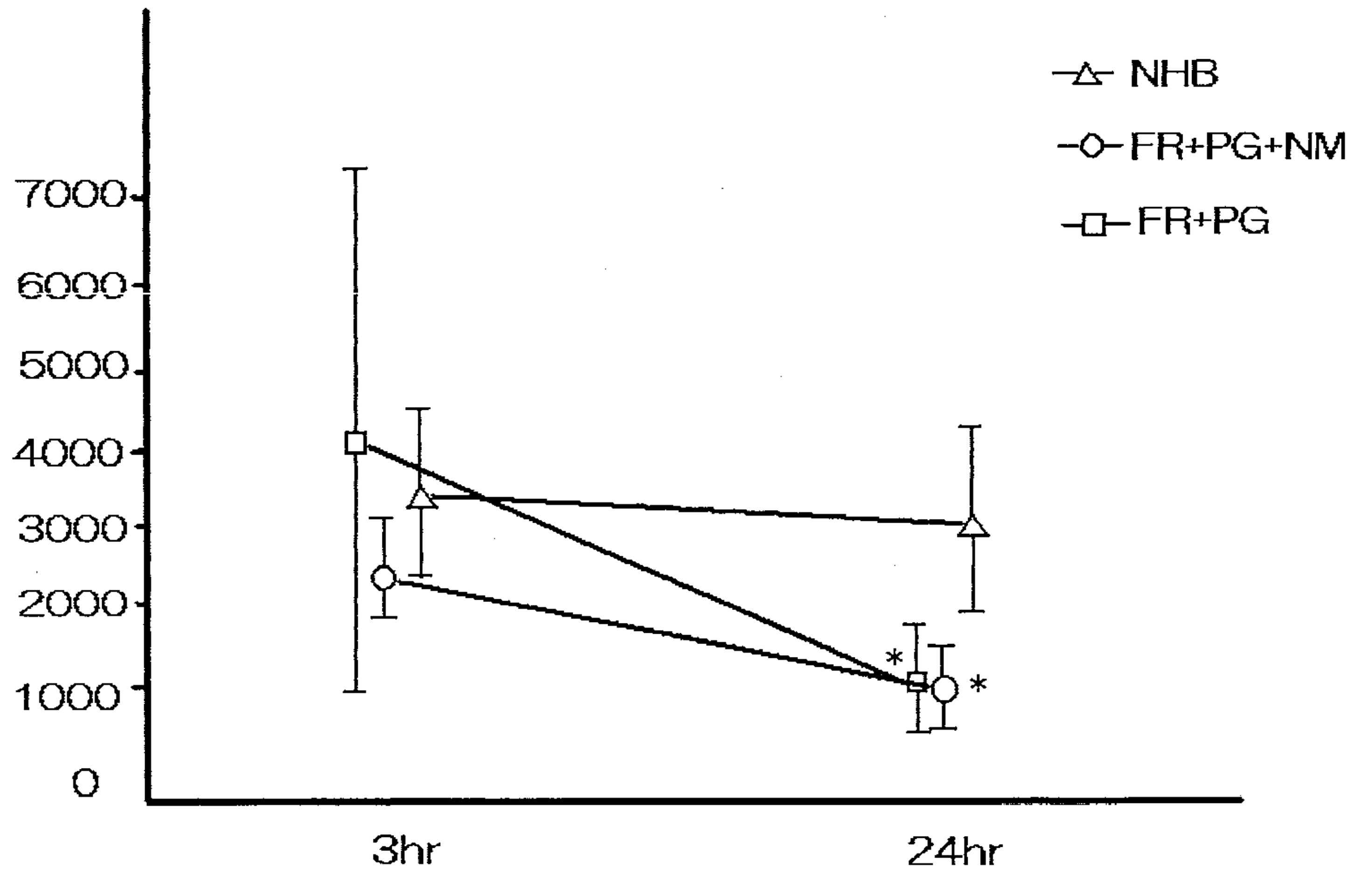
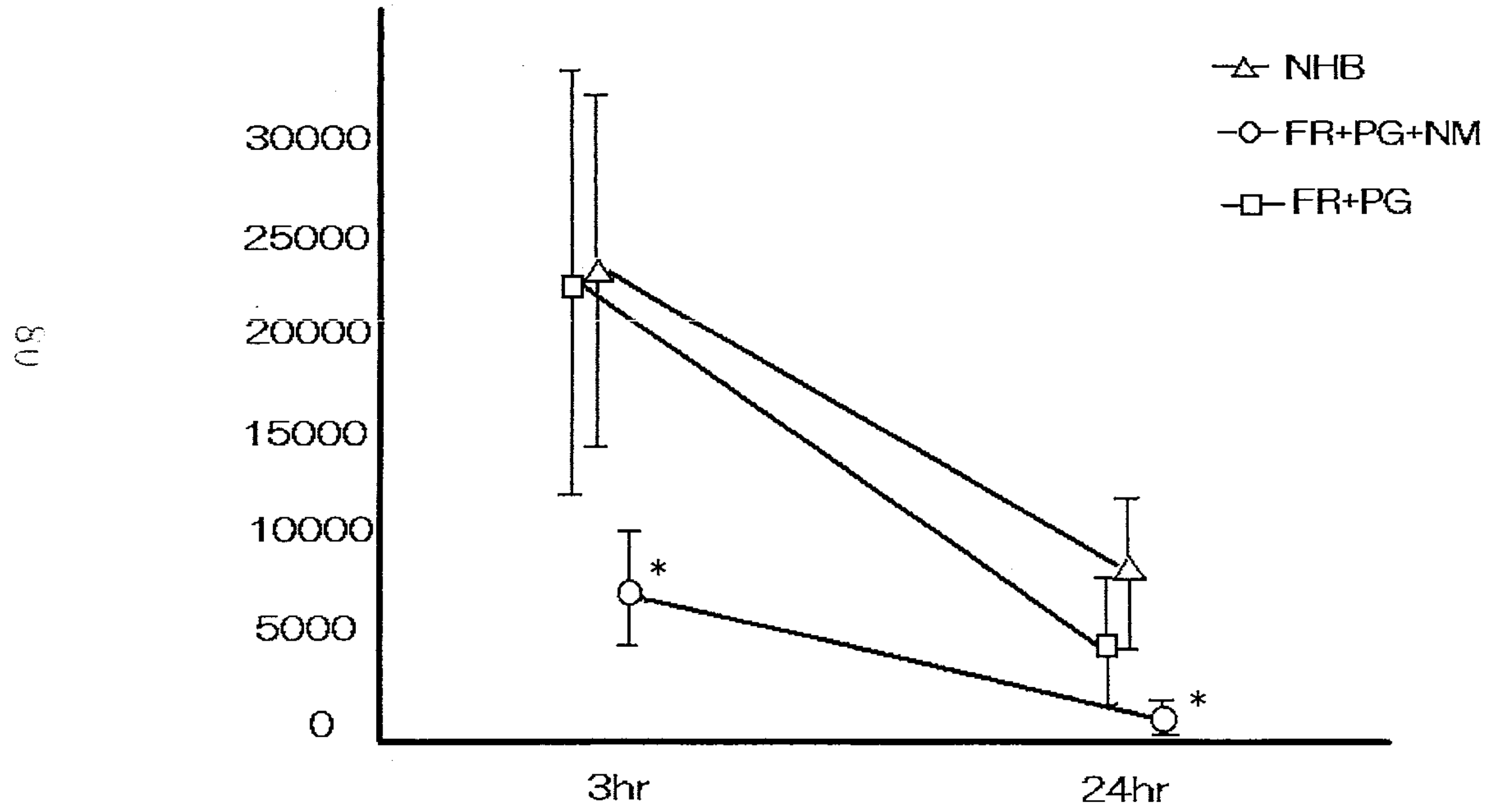


図3　血清ALT



＊ p＜0. 05 vs NHB

図4　血清LDH



＊ $p<0.05$ vs NHB

表　各群の死戦期、無肝期、グラフト総虚血時間

	死戦期	無肝期	総虚血時間
FR+PG+NM	11.3±1.2	15.4±0.7	114.6±1.1
FR+PG	10.4±0.9	15.3±0.9	114.6±1.2
FR+NM	10.8±1.1	15.7±1.0	114.4±1.1
PG+NM	10.4±1.1	15.6±1.1	115.3±1.3
FR	10.6±1.2	15.4±1.2	115.1±1.2
PG	10.5±1.1	15.4±0.8	115.4±1.0
NM	10.4±1.1	16.2±0.8	115.1±1.4
NHB(Control)	10.4±1.1	15.6±0.8	114.7±1.0

単位:分

課題４：臨床応用を前提とした大動物を用いた死戦期を伴う心臓死

ドナー肝の障害に対する薬剤の効果の検討

豚を用いた移植実験

1） 要約

【目的】心停止ドナーからの肝移植時に問題となる阻血再灌流障害を、臨床応用可能な薬剤を投与することによって抑制し、心停止肝移植を成功に導くことを目的とした．そのためブタを用いた心停止ドナーからの肝移植実験を行い、薬剤投与の効果を検討した．

【方法】ブタを用い以下の５つの群に分け実験を行った．（１）Control 群：以下に述べる薬剤を用いず心停止肝移植を行った群．（２）FR 群：再灌流障害の軽減を目的として、TNF-αおよびIL-1βの阻害剤であるFR167653（FR）の投与を行い心停止肝移植を行った群．（３）PG 群：温阻血障害発生の予防を目的として、プロスタグランジン E_1（PG）の投与を行い心停止肝移植を行った群．（４）FRPG 群：FR、PG の双方を投与し心停止肝移植を行った群．（５）HBD 群：positive control として心拍動下肝移植を行った群．

【結果】PG 投与によって温阻血障害が抑制され、FR 投与によって再灌流障害が軽減されたが、これらの薬剤は単独の投与では生存率の向上は得られなかった．PG と FR の双方を投与することによって心拍動下肝移植と同等の生存率が得られた．

【結論】心停止ドナーからの肝移植では心拍動下の移植と異なり、温阻血障害の存在とこれに引き続くより高度の再灌流障害が問題となる．本研究ではこれらが臨床応用可能な薬剤によって制御可能であることを示唆し、その結果、心停止肝移植を安全に行うことが可能であると示唆された．

2） 研究背景

肝移植におけるドナー不足は世界的に深刻な問題である．近年まで、移植に供される肝グラフトは心拍動下に摘出されたもののみが適すると考えられていたが、もし心停止ドナーからの肝移植が可能であれば、施行される肝移植の例数は増加し、ドナー不足の解消の一助となると考えられる．臨床においては、いくつかの施設で心停止肝移植が試みられているが、心停止ドナーからの肝グ

ラフトは脳死あるいは生体ドナーからのそれに比べて高頻度に primary graft non-function を惹起すると言われている[1) 2) 3)]. 当教室ではこれまでに、心停止ドナーからの肝グラフトでは再灌流後に微小循環障害をきたし、その結果として primary graft non-function が生じることを示した[4) 5)]. また、微小循環障害は肝類洞内皮細胞障害と類洞の狭小化が原因となっており、類洞内皮細胞障害は Kupffer 細胞を除去することで、類洞の狭小化はメシル酸ナファモスタットリンスを用いることでそれぞれ抑制可能であることを示した[6)]. しかしながら、実際の臨床の場において Kupffer 細胞を除去した肝グラフトを用いて移植を行うことは困難であると考えられ、これに代わって微小循環障害を抑制し心停止肝移植を成功に導く方法を見いだす必要があると考えられた. Kupffer 細胞が類洞内皮細胞障害を惹起する機序の一環として、当教室では TNF-α が Kupffer 細胞による活性酸素産生を誘導しこれが好中球の集積を来すことを示した[7)]. また、心停止ドナーからの肝グラフトでは、再灌流の際に心拍動下に摘出したグラフトに比し有意に灌流液中の IL-1β が増加しており Kupffer 細胞除去によって抑制されることも示した[8)]. 以上より、TNF-α および IL-1β の産生阻害剤である FR167653 は[9)]、心停止肝移植の際に生じる微小循環障害に対して Kupffer 細胞除去と同程度の効果が期待できると考えられた.

3） 研究目的

臨床応用可能と思われる薬剤を用いて、心停止ドナーからの肝移植を成功に導く方法を開発する.

4） 研究方法

1） 実験動物

体重 20-30 kg の Landrace ブタを用い、以下の５群に分け移植実験を行った.

（1）Control 群（n=4）：以下の薬剤を使用せず心停止肝移植を行った. 再灌流

直前のリンス液にはラクテートリンゲル液を使用した.

（２）FR 群（n=4）: TNF-α および IL-1β の産生阻害剤である FR167653（FR、藤沢製薬）1.0 mg/kg/h をドナーおよびレシピエントに持続静脈内投与した. リンス液にはメシル酸ナファモスタット（NM）リンス（表１，鳥居薬品）を使用した.

（３）PG 群（n=4）: 温阻血障害予防を目的としてプロスタグランジン E_1（PG、小野薬品）20 ng/kg/min をドナーおよびレシピエントに持続静脈内投与した. リンス液には NM リンスを使用した.

（４）FRPG 群（n=4）: FR 群と同様に FR を、PG 群と同様に PG を投与した. リンス液には NM リンスを使用した.

（５）HBD 群（n=4）: 薬剤を使用せず心拍動下に肝グラフトを摘出し移植を行った.

実験に使用したブタは手術の 24 時間前より絶食とし水分のみを与え、ニフレック 1000 ml にて前処置を行った. 各リンス液は４℃に保って使用した.

２） ドナー手術

ケタミンと硫酸アトロピンを投与した後に耳静脈を確保し、イソフルレンと酸素の吸入下に気管切開を行い、イソフルレンと筋弛緩剤による全身麻酔下に以下の手術操作を行った. 中心静脈のモニタリング用に外頸静脈を確保、動脈圧のモニタリング用に総頸動脈を確保した. 開腹操作終了後、HBD 群以外ではメチルプレドニゾロン 10 mg/kg およびウリナスタチン（UTI、持田製薬）30,000 U/kg を静脈内投与した. ヘパリン 300 U/kg を静脈内投与した後、脾静脈、総胆管および腹部大動脈にカニュレーションし、十分量の筋弛緩剤投与後に人工呼吸器を外して心停止を誘導した. 心停止に至るまでの間中心静脈圧が 14 cmH20 以下になるように総腸骨静脈より適宜脱血した. 心電図にて心停止を確認し 10 分間待機した後、脾静脈および腹部大動脈から４℃に冷却した University of Wisconsin 液（UW 液）500 ml で肝グラフトを灌流冷却した. 肝グラフトを摘出し肝動脈より UW 液 50 ml を注入、氷冷下に３時間単純浸漬保存した. HBD 群では死戦期を経ず同様の操作を行った.

３） レシピエント手術

ドナーと同様に全身麻酔を施し以下の手術操作を行った. 動脈圧モニタリングのために右頸動脈に、採血および薬剤投与のために外頸静脈にカニュレーションを行った. 開腹し肝を摘出したが、無肝期の間の門脈ならびに肝下下大静

脈の血流はバイオポンプ（Medtronic Bio-Medicus, Inc., USA）を用いて左外頚静脈にバイパスした．この際抗凝固を目的として回路内にNM 1 mg/kg/hをバイオポンプ停止まで持続投与した．肝移植は同所性に行い 4-0 Prolene を用いた連続縫合で肝上下大静脈、門脈、肝下下大静脈の順に端々吻合した後、最後に 8-0 Prolene を用いた結節縫合で肝動脈の端々吻合を行った．門脈吻合に先立って、各リンス液を用いて肝グラフト内のUW液と置換した．門脈吻合終了後、門脈血を用いて肝グラフトを再灌流した．最後に胆汁外瘻を作成し閉腹した．

術後は高カロリー液を用いた中心静脈栄養管理とし、H2 ブロッカー、抗生物質および免疫抑制剤としてFK506（藤沢製薬）0.1 mg/kg/dayを投与した．

4）　検討項目

肝組織のサンプリングはドナー開腹時、ドナー心停止7分後および再灌流後1時間の時点で行った．

（1）肝組織中Energy charge

サンプル採取後直ちに液体窒素内でフリーズクランプし、-80℃で凍結保存した後 0.5N 過塩素酸を用いて抽出し、液体クロマトグラフィー（Jasco HPLC Analyzer system；日本分光）にて ATP、ADP および AMP を測定した．溶出には 60 mM のリン酸バッファー（pH 6.0）を用い、流速 1.0 ml/min、室温下で行った．Atkinson と Chapman の式により Energy charge を算出した[10]．

（2）肝組織中ミトコンドリア proton ATPase 活性

ミトコンドリア proton ATPase 活性の測定は瀬谷らの方法で行った[11]．まずミトコンドリア画分をカリウム緩衝液に加え、シアニン色素 Dis3C を添加して生ずる蛍光強度を蛍光強度計（Jasco FP-777；日本分光）を用いて測定し基準電位とした．アンチマイシン 250 ng を加えて電子伝達系を抑制した後、ATP 2 μmol を加え生じた蛍光強度の変化を基準電位で除したものを proton ATPase 活性（%）として表した．

（3）組織学的検討

光顕標本は検体採取後10%中性ホルマリンにて固定し、ヘマトキシリン-エオジン染色により作成し観察した．電顕標本は、採取後速やかに 1 mm 角に細切し、2.5%グルタールアルデヒドおよび2%パラフォルムアルデヒド含有 0.1 M カコジレートバッファー（pH 7.3）にて前固定した．1%オスミウムにて後固定した後、アルコールおよび酸化プロピレンを用いて段階的に脱水し、エポキシ樹脂に包埋した．超薄切切片を透過電子顕微鏡（JEM-1010；日本光電）を用いて観

察した.

（4）血清生化学検査

血清 AST、LDH を酵素比色方にて測定した (AST: : ヤトロン、LDH : 和光純薬).

5） 統計学的検討

データは mean±SD で表した. 検定は one-factor ANOVA と post-hoc test の方法により、P<0.05 を有意差ありとした.

5） 研究結果

1） 移植成績

平均温阻血時間は 31.2±2.9 分で、HBD 群を除く 4 群で統計学的有意差を認めなかった. 平均冷保存時間は 3.0±0.3 時間で、5 群間に有意差を認めなかった. Control 群と FR 群では全例が 24 移植後 24 時間以内に死亡し、解剖時に多量の血性腹水を認めた. PG 群では 2 例が移植後 24 時間以内に死亡し、解剖時の所見は Control 群、FR 群と同様であった. 4 例中 2 例が 7 日間生存した. FRPG 群および HBD 群では全例が 7 日間生存した（表 2）.

2） 血清生化学検査

血清 AST および LDH は HBD 群以外の群で再灌流後に上昇を認め、特に FR 群で顕著であった. AST、LDH は術後第 1 病日をピークにその後漸減し、術後第 7 病日には血清 AST 値は正常に復した. 各々の時点で各群間に統計学的有意差を認めなかった（図 1）.

3） 肝組織中 Energy charge

図 2 に肝組織中 Energy charge の経時的変化を示す. 心停止移植を行った 4 群では、心停止時には開腹時の値の 50%以下に有意に低下しており、Control 群以外では再灌流後に開腹時の 50-85%の値にまで復帰した. FR 群では PG 群、FRPG 群に比して再灌流後の Energy charge 値の再上昇率が低い傾向があった.

4） 肝組織中ミトコンドリア proton ATPase 活性

図 3 に肝組織中ミトコンドリア proton ATPase 活性の経時的変化を示す. 心停止時の ATPase 活性は、Control 群、FR 群および PG 群で開腹時より低値を示したが、FRPG 群では有意な低下は認めなかった. 再灌流後には Control 群、FR

群およびPG群では心停止時より更に低値となったが、FRPG群およびHBD群では開腹時と同等の値を示し、他の3群に比して有意に高値であった．

5）　組織学的検討

（1）光顕所見

心停止時には、zone 1の類洞腔はPG群およびFRPG群で広く保たれていたが、Control群およびFR群では類洞腔は狭小化していた（図4）．再灌流後には、Control群、FR群およびPG群で類洞腔の狭小化と出血、zone 2での肝細胞の部分壊死の像が認められた．これらの変化はFRPG群およびHBD群では認められなかった（図5）．

（2）電顕所見

心停止時、PG群およびFRPG群では肝細胞に虚血空胞が散見されることを除き類洞内皮細胞、肝細胞ともにほぼ正常な像を呈したが、Control群およびFR群では類洞内皮細胞の破壊と肝実質細胞の細胞成分の類洞腔への流出が認められた（図6）．再灌流後には、FRPG群およびHBD群では類洞内皮細胞、肝細胞ともによく保たれていたが、他の3群では類洞腔およびDisse腔は完全に破壊されており肝細胞の微絨毛もほとんど認められなかった．実質細胞の細胞成分の類洞腔への流出、Disse腔内に赤血球が入り込んでいる像が認められた（図7）．

6）　考察

肝移植における待機患者の数は増加し続けており、また待機時間も長期化している[12]．移植医は心停止ドナーのようなこれまで適応外とされていたドナーにまでドナーソースを求めなければならず、いくつかの施設では心停止肝移植が試みられている．しかしながら心停止肝移植はprimary graft non-functionを高率に引き起こすため、あまり行われていないのが現状である．当教室では心停止肝移植を成功させるための最大の障害となるのが、肝微小循環障害であることを示し、この微小循環障害は類洞内皮細胞障害と類洞腔の狭小化という二つの主要な因子によることを示した．さらに当教室では、Kupffer細胞除去が類洞内皮細胞障害を軽減し、NMリンスを用いることで類洞腔の狭小化が改善されることを示した[6]．この報告ではKupffer細胞を除去するためにリポソーム封入クロドロネート（L-DMDP）を用いたが、肝摘出の42時間前にL-DMDPを投与する必要があった．この投与法では臨床応用することは困難と考えられ、

Kupffer 細胞の機能を制御する他の方法を模索しなければならなかった．本研究では、TNF-αおよび IL-1βの産生阻害剤である FR を用い、これが心停止肝移植の再灌流時において類洞内皮細胞障害を軽減することを明らかにした．

当教室では、Kupffer 細胞の機能を抑制することが、心停止ドナーからの肝移植を成功に導く重要な因子であることを示した．死戦期には腸管でのサイトカイン産生が亢進し、それが肝グラフトに流入することで Kupffer 細胞がプライミングされ、プライミングされた Kupffer 細胞は冷保存によりさらに活性を上昇させる[13]．再灌流後 TNF-αが Kupffer 細胞からの活性酸素産生を誘導し、この活性酸素がメディエーターとなって好中球の集積を来す[7]．好中球の集積は微小循環障害を惹起すると考えられ[14]，接着、浸潤した好中球はさらに活性酸素やサイトカイン、プロテアーゼなどの多くのメディエーターを放出し炎症反応を増幅させる． 当教室のこれまでの研究から、TNF-αの産生を阻害することによって Kupffer 細胞の機能を抑制し、上に述べたような再灌流に引き続いて生じる連鎖反応を軽減できることが示唆された[7]．また当教室では心停止ドナーからの肝グラフトにおいて、IL-1β産生が有意に増加していることも示した[8]．これらのことから本研究では、Kupffer 細胞を除去する代わりに FR を投与することによって微小循環障害を軽減しようと考えた．本研究において、再灌流後の電顕所見で、PG 群では肝グラフトの類洞内皮細胞の断裂、剥離が著名であったが FRPG 群では類洞内皮細胞障害ははるかに軽度であった．この結果から、心停止肝移植における類洞内皮細胞障害の軽減には、TNF-α産生を阻害して Kupffer 細胞の機能を抑制することが重要であると示された．

心拍動下に摘出された肝グラフトと心停止ドナーからのグラフトの相違は、グラフトにかかる温阻血の有無である．心停止ドナーからの肝グラフトの viability は摘出前の温阻血によって損なわれると考えられる．高田らは、特に前処置を施されず 60 分間の温阻血を被った肝グラフトでも移植可能であると報告している[15]．本研究における温阻血時間は約 30 分であるが、Control 群における移植成績は高田らの報告よりも悪かった．この成績の差は心停止モデルの設定にあると考えられる．高田らの報告をはじめ多くの温阻血に関する研究で用いられている心停止ドナーのモデルは、塩化カリウムで心停止を誘導しているものが多い．このようなモデルは低酸素、低 pH、低血圧、腸管のうっ血といった、いわゆる死戦期を経ていない心停止ドナーのモデルである．当教室では、心停止の直前に腸管から放出されるサイトカインが、冷保存後の再灌流障害を

増悪させることを示した (Miyagi et al. Role of arachidonic acid cascade in warm ischemia-reperfusion injury of liver grafts from non-heart-beating rats、投稿中). また、温阻血時には細胞内カルシウムの増加が生じ、これが細胞障害を引き起こす反応の第一段階であるといわれている[16、17]. PG は細胞内カルシウム濃度の上昇を遅延させ細胞膜の bleb 生成を抑制すると報告されている[18]. 本研究において、摘出直前の電顕所見で PG を投与されない肝グラフトでは類洞内皮細胞障害が著明であったが、PG を投与されたグラフトでは障害は軽度であった. これらのことから、心停止の直前に類洞内皮細胞は障害を受け、これを予防することが肝要であると考えられた.

7) 結論

本研究において、PG 投与によって温阻血時の類洞内皮細胞障害が軽減され、FR 投与によって再灌流時の類洞内皮細胞障害が軽減されていた. この両者の併用によって移植成績が向上し、臨床の心停止肝移植においても有用な治療法になりうると考えられた.

8) 文献

1) Casavilla A, Ramirez C, Shapiro R, Nghiem D, Miracle K, Bronsther O, Randhawa P, Broznick B, Fung JJ, Starzl T. Experience with liver and kidney allografts from non-heart-beating donors. Transplantation 1995; 59: 197-203

2) D'Alessandro AM, Hoffmann RM, Knechtle SJ, Eckhoff DE, Love RB, Kalayoglu M, Sollinger HW, Belzer FO. Successful extrarenal transplantation from non-heart-beating donors. Transplantation 1995; 59: 977-982

3) Reich DJ, Munoz SJ, Rothstein KD, Nathan HM, Edwards JM, Hasz RD,

Manzarbeitia CY. Controlled non-heart-beating donor liver transplantation (A successful single center experience, with topic update). Transplantation 2000; 70: 1159-1166

4) Endoh T, Ohkohchi N, Katoh H, Seya K, Satomi S, Mori S, Nakamura K. Graft conditioning of liver in non-heart-beating donors by an artificial heart and lung machine in situ. Transplant Proc 1996; 28: 110-115

5) Tsukamoto S, Ohkohchi N, Endoh T, Seya K, Satomi S, Mori S. Procurement of liver grafts by an artificial heart-lung machine using leukocyte-depleted washed red blood cells in non-heart-beating donors. Transplant Proc 1996; 28: 197-200

6) Tsukamoto S, Ohkohchi N, Fukumori T, Orii T, Asakura T, Takayama J, Shibuya H, Kato H, Satomi S. Elimination of Kupffer cells and nafamostat mesilate rinse prevent reperfusion injury in liver grafts from agonal non-heart-beating donors. Transplantation 1999; 67: 1396-1403

7) Shibuya H, Ohkohchi N, Tsukamoto S, Satomi S. Tumor necrosis factor-induced, superoxide-mediated neutrophil accumulation in cold ischemic/reperfused rat liver. Hepatology 1997; 26: 113-120

8) Oikawa K, Ohkohchi N, Sato M, Satomi S. The effects of the elimination of Kupffer cells in the isolated perfused liver from non-heart-beating rat. Transpl Int 2000; 13 [Suppl 1]: S573-S579

9) Yamamoto N, Sakai F, Yamazaki H, Nakahara K, Okuhara M. Effect of FR167653, a cytokine suppressive agent, on endotoxin-induced disseminated intravascular coagulation. Eur J Phamachol 1996; 314: 137-142

10) Atkinson DE, Chapman AG. The adenylate energy charge in the study of enzymes in vitro. Methods Enzymol 1979; 55: 229-235

11) Seya K, Ohkohchi N, Watanabe N, Shibuya H, Taguchi Y, Mori S. Rapid fluorometric assay for mitochondrial proton adenosine triphosphatase activity for assessment of viability of liver graft tissue. J Clin Lab Anal 1994; 8: 418-423

12) Bismuth H. The need for a consensus agreement on indications of liver transplantation. Hepatology 1994; 20: 1s-2s

13) Mochida S, Arai M, Ohno A, Masaki N, Ogata I, Fujiwara K. Oxidative stress in hepatocytes and stimulatory state of Kupffer cells after reperfusion differ between warm and cold ischemia in rats. Liver 1994; 14: 234-240

14) Ferguson D, McDonagh PF, Biewer J, Paidas CN, Clemens MG. Spatial relationship between leukocyte accumulation and microvascular injury during reperfusion following hepatic ischemia. Int J Microcirc Clin Exp 1993; 12: 45-60

15) Takada Y, Taniguchi H, Fukunaga K, Yuzawa K, Otsuka M, Todoroki T, Iijima T, Fukao K. Hepatic allograft procurement from non-heart-beating donors: limits of warm ischemia in porcine liver transplantation. Transplantation 1997; 63: 369-373

16) Clavien P, Harvey P, Strasberg S. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. Transplantation 1992; 53: 957-978

17) Gasbarrini A, Borle AB, Farghali H, Bender C, Francavilla A, van Thiel D. Effect of anoxia on intracellular ATP, Na+i , Ca2+i, Mg2+i, and cytotoxicity in rat hepatocytes. J Biol Chem 1992; 267: 6654-6663

18) Kishimoto S, Sakon M, Umeshita K, Miyoshi H, Taniguchi K, Meng W, Nagano H, Dono K, Ariyosi H, Nakamori S, Kawasaki T, Gotoh M, Monden M, Imajoh-Ohmi S. The inhibitory effect of prostaglandin E1 on oxidative stress-induced hepatocyte injury evaluated by calpain-μ activation. Transplantation 2000; 69: 2314-2319

Figure legend

図1：血清 AST 値 (a) および血清 LDH (b)．HBD 群以外の全群で AST 値、LDH 値ともに再灌流後に上昇しており、特に FR 群で顕著であった．AST 値、LDH 値は移植後第1病日まで高値を示し、その後漸減した．AST 値は移植後第7病日には正常値となった．各時点で群間に統計学的有意差を認めなかった．（Laparotomy：レシピエント開腹時、Reperfusion：再灌流 5 分後、POD：移植後病日）

図2：肝組織中 Energy charge の経時的変化．心停止移植を施行した群では心停止時に有意な低下を認め、Control 群以外では再灌流後に回復した．再灌流後の Energy charge は各群に統計学的有意差を認めなかった．*P<0.05 vs 開腹時（Laparotomy：ドナー開腹時、Cardiac arrest：心停止 7 分後、After reperfusion：再灌流 1 時間後）

図3：肝組織中ミトコンドリア proton ATPase 活性の経時的変化．Control 群、FR 群および PG 群では ATPase 活性は心停止時に低下したが、FRPG 群および HBD 群では低下しなかった．再灌流後には Control 群、FR 群および PG 群ではさらに活性が低下したが、FRPG 群および HBD 群では開腹時と同等の値を示し他の 3 群より有意に高値であった．*P<0.05 vs FRPG 群、**P<0.05 vs FRPG 群および HBD 群、***P<0.05 vs HBD 群（Laparotomy：ドナー開腹時、Cardiac arrest：心停止 7 分後、After reperfusion：再灌流 1 時間後）

図4：心停止 7 分後の光顕写真（ヘマトキシリン-エオジン染色）．Zone 1 の類洞腔は PG 群および FRPG 群で広く保たれており、Control 群および FR 群では狭小化している．

図5：再灌流 1 時間後の光顕写真（ヘマトキシリン-エオジン染色）．Control 群、FR 群および PG 群では類洞腔は狭小化しており出血が認められ、zone 2 に部分

的な肝細胞壊死も認められる．これらの変化はFRPG群およびHBD群では認められない．

図6：心停止7分後の電顕写真．PG群およびFRPG群では類洞内皮細胞はほぼ正常であるが、Control群およびFR群では類洞内皮の変性が認められ細胞成分の類洞内への流出が認められる．Scale bar = 2μm

図7：再灌流1時間後の電顕写真．FRPG群およびHBD群では類洞内皮細胞、肝実質細胞ともによく保たれているが、Control群、FR群およびPG群では類洞腔とDisse腔は完全に破壊されており肝細胞の微絨毛もほとんど認められない．また、細胞成分の流出とDisse腔内に赤血球が認められる．Scale bar = 2μm

Table 1 Recipient survival after LTx

group	survival	cause of death
FR group	< 24 hours	primary graft failure
(n = 4)	< 24 hours	primary graft failure
	< 24 hours	primary graft failure
	< 24 hours	primary graft failure
PG group	< 24 hours	primary graft failure
(n = 4)	< 24 hours	primary graft failure
	> 7 days	sacrifice
	> 7 days	sacrifice
FRPG group	> 7 days	sacrifice
(n = 4)	> 7 days	sacrifice
	> 7 days	sacrifice
	> 7 days	sacrifice

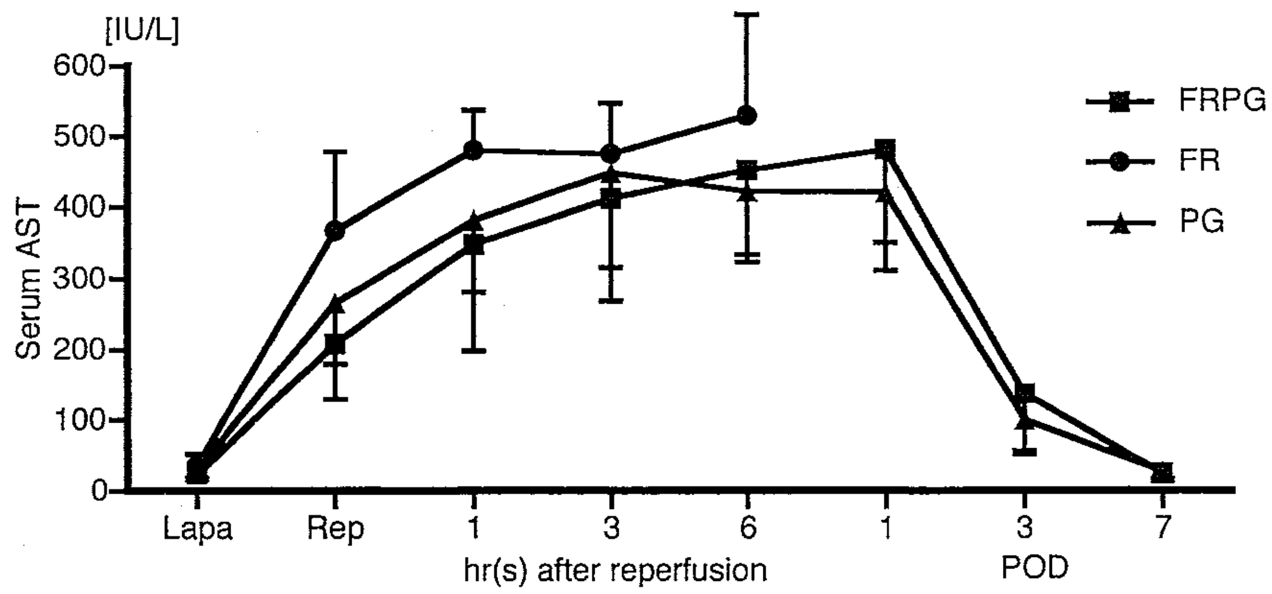


Figure 1 a

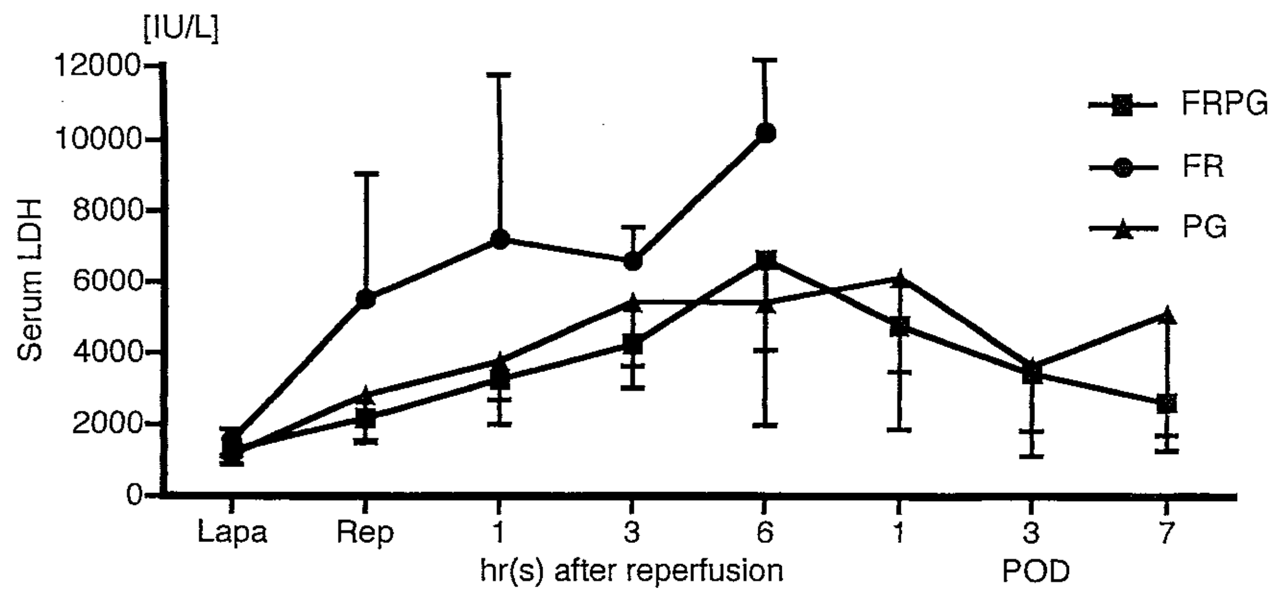


Figure 1b

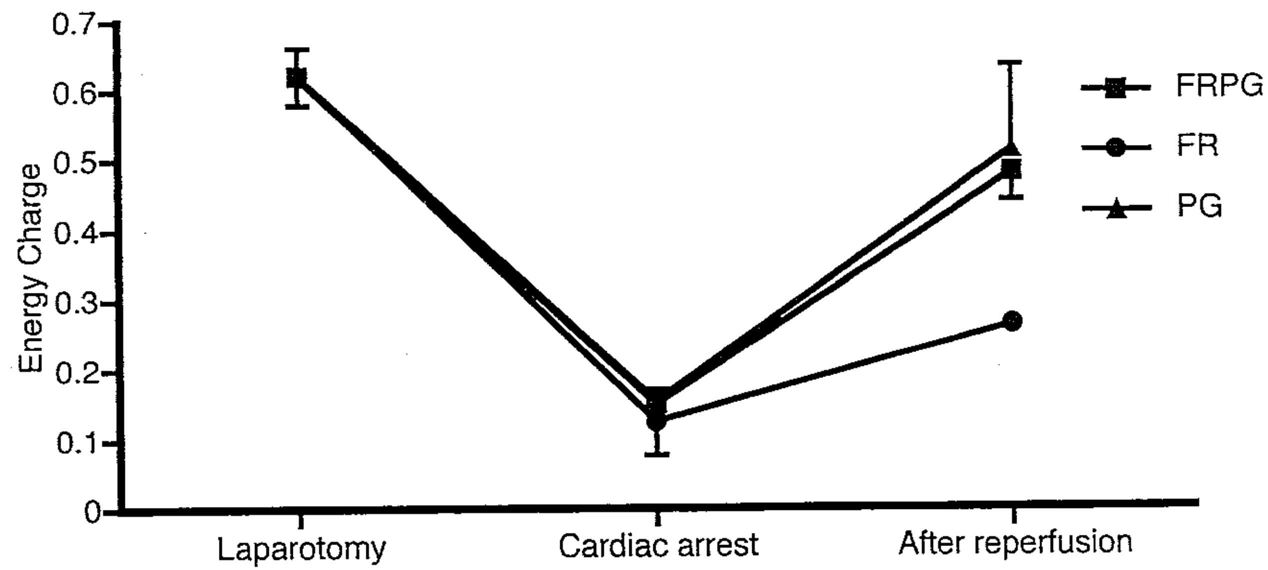


Figure 2

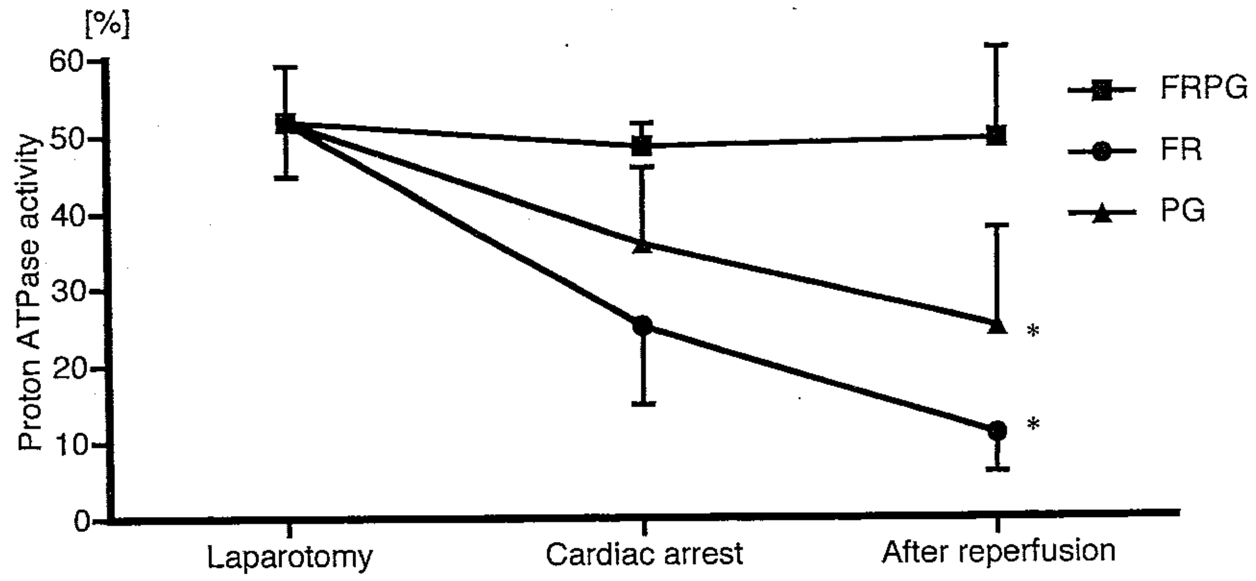


Figure 3

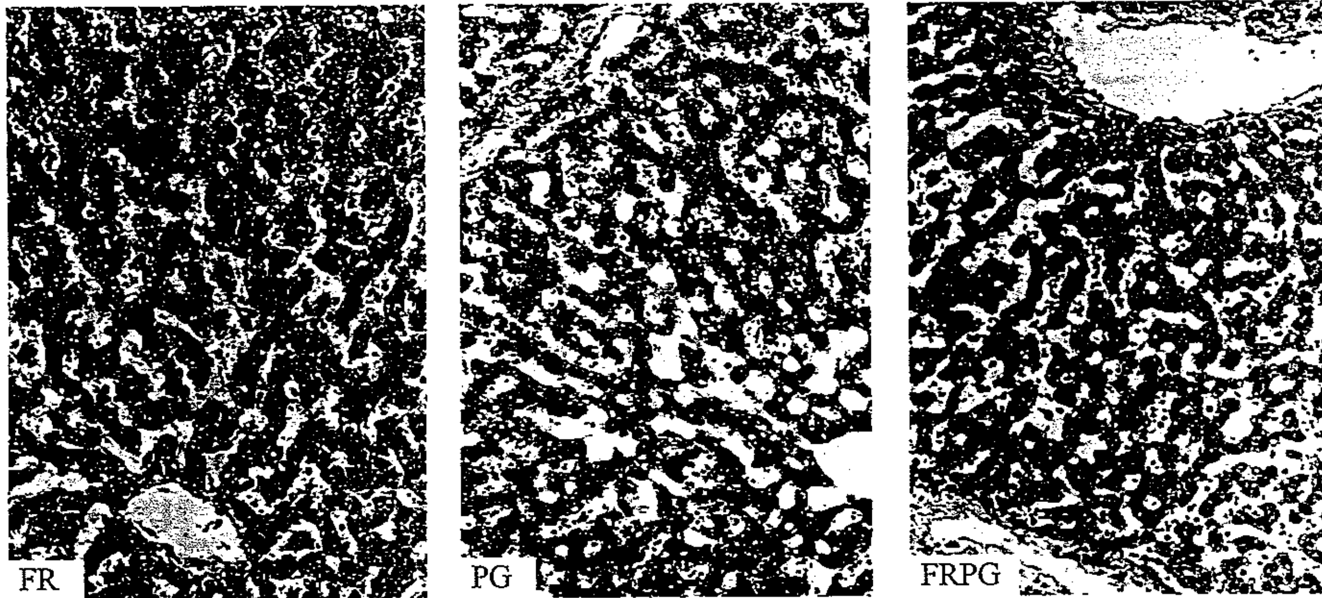


Figure 4

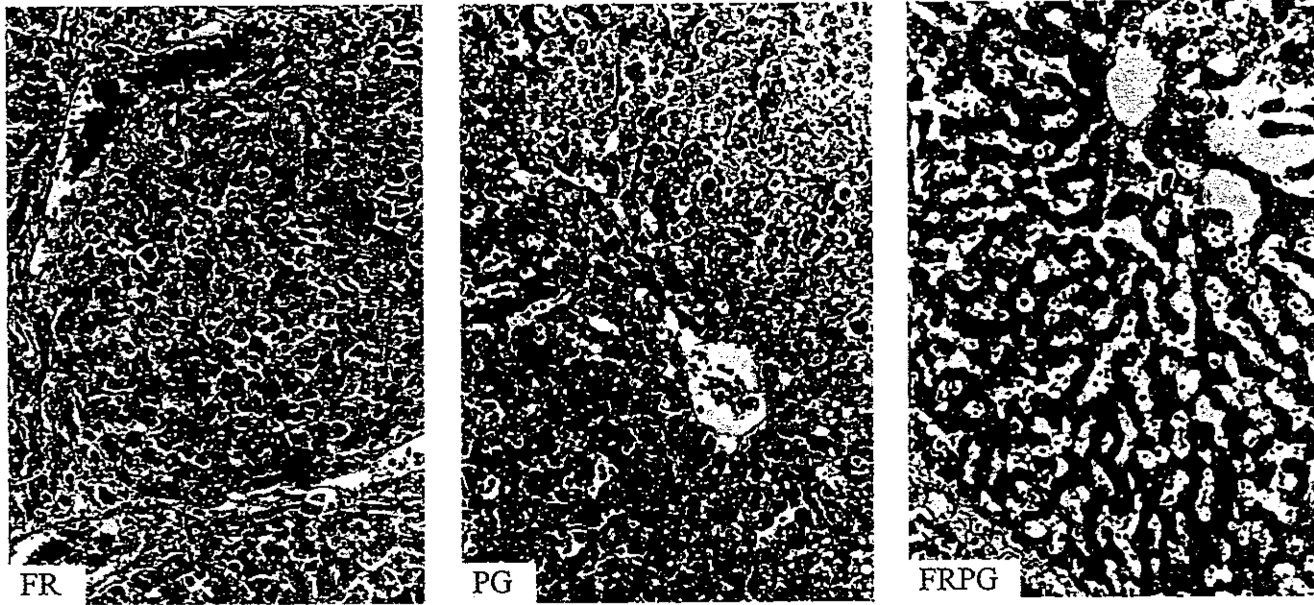


Figure 5

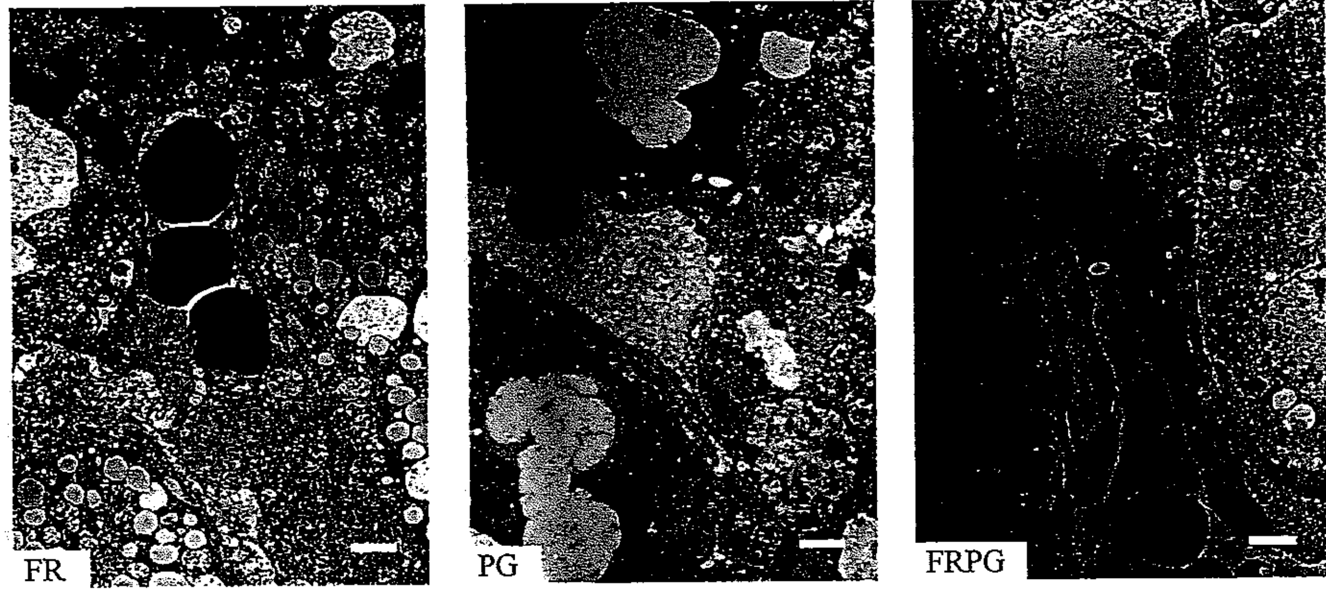
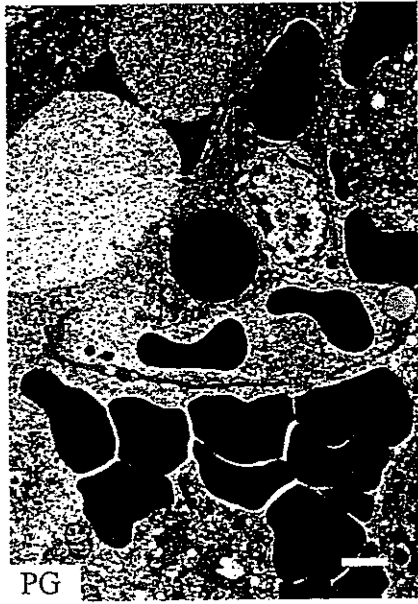


Figure 6



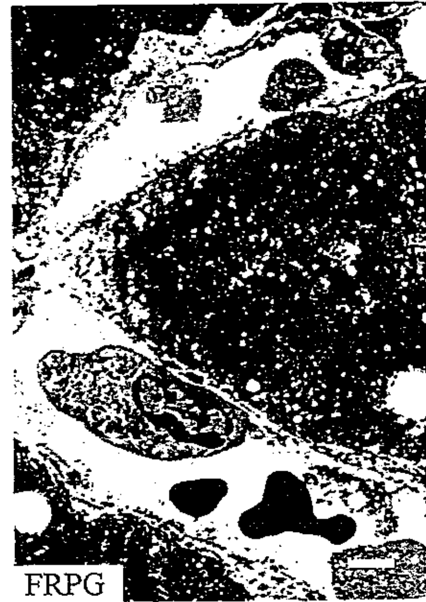


Figure 7

ELSEVIER

New Strategy for Liver Transplantation From Non–Heart-Beating Donors

M. Sato, N. Ohkohchi, S. Tsukamoto, T. Orii, N. Koyamada, T. Asakura, J. Takayama, Y. Enomoto, M. Goto, M. Usuda, S. Miyagi, A. Okada, and S. Satomi

THE shortage of donors has become a serious problem in liver transplantation (LTx). LTx from controlled non-heart-beating donors (NHBD) has been attempted. However, it has been reported that the grafts from NHBD develop primary graft nonfunction more often than those from heart-beating donors. The aim of this study was to discover a safer method to prevent ischemia and reperfusion injuries in liver grafts from NHBD.

MATERIALS AND METHODS

Landrace white pigs, weighing 20 to 30 kg, were allocated to three groups as follows: (i) *Control group* (n = 5): To prevent warm ischemic injury, donor pigs were administered 1 mg/kg FK 506 (Fujisawa Pharmaceutical Co., Japan) intramuscularly 24 hours before surgery, and 100 ng/kg per minute of OP2507, a prostaglandin (PG) I_2 analogue (Ono Pharmaceutical Co., Japan) intravenously during surgery. (ii) *LD group* (n = 5): Donor pigs were intravenously administered liposome-encapsulated dichloromethylene diphosphonate (L-DMDP) 42 hours before liver procurement to eliminate Kupffer cells and were given FK 506 and OP2507 in the same manner as in the control group. (iii) *FR group* (n = 4): To prevent warm ischemic injury, 20 ng/kg per minute of PGE_1 (Ono Pharmaceutical) and to prevent reperfusion injury, 1.0 mg/kg per hour of FR167653 (FR), which is a dual inhibitor of TNF-α and IL-1β production (Fujisawa Pharmaceutical), were administered intravenously during surgery. Both donors and recipients were intravenously administered 30,000 U/kg of ulinastatin (UTI; Mochida Pharmaceutical Co, Japan).

Donor Operation

Laparotomy was performed under general anesthesia. Initially, 300 U/kg body weight of heparin and 250 mg of methylprednisolone were administered intravenously. After the splenic vein and the abdominal aorta were cannulated, the respiration was stopped with pancronium bromide. The central vein pressure was measured and kept below 14 cm H_2O until cardiac arrest was confirmed on the electrocardiogram. Ten minutes after the cardiac arrest, perfusion of the liver with cold solutions was initiated using Ringer's lactate in the control group and the LD group, and University of Wisconsin (UW) solution in the FR group. The liver was preserved in UW solution at 4°C for 3 hours.

Recipient Operation

After laparotomy, the liver was dissected free and removed. During the anhepatic phase, a bio-pump was employed for veno-venous bypass. Then the graft liver was implanted orthotopically. Immediately before the anastomosis of the portal vein, the graft liver was flushed with 400 mL of nafamostat mesilate (NM) rinse to remove the UW solution.

Examination Items

Energy charge of the liver tissue was measured to evaluate ATP synthesis in the graft. Electron microscopy was also carried out to observe morphological changes.

RESULTS

In all groups, the values of hepatic energy charge at cardiac arrest decreased 20% to 40% from the values at laparotomy. At 1 hour after reperfusion, the values recovered 75% to 90% of the initial values (Fig 1).

In the morphological study, electron microphotographs after laparotomy showed disruption and detachment of sinusoidal lining cells (SLCs) in the control group but well-preserved SLCs and sinusoidal structure in both the LD and the FR groups.

All recipients in the control group survived fewer than 7 days, but four of the five recipients in the LD group and all four recipients in the FR group survived more than 7 days (Table 1).

DISCUSSION

Our NHBD model is similar to a clinically controlled NHBD. We previously used this NHBD model for successful LTx by maintaining microcirculation after reperfusion.[1] In our previous study, L-DMDP was administered 42 hours before the liver procurement to avoid microcirculatory disturbance and to eliminate Kupffer cells in the liver graft. However, in clinical practice, it is difficult to administer drugs long before the procurement. In this study, we performed LTx from NHBD using only drugs that could be administered during actual clinical operations.

From Graduate School of Medicine, Tohoku University; and the Department of Surgery (N.O.), Institute of Clinical Medicine, Sendai, Japan.

Address reprint requests to M. Sato, Division of Advanced Surgical Science and Technology, Graduate School of Medicine, Tohoku University, 1-1 Seiryo-machi, Aoba-ku, Sendai, 980-8574 Japan.

© 2002 by Elsevier Science Inc.
360 Park Avenue South, New York, NY 10010-1710

0041-1345/02/$–see front matter
PII S0041-1345(02)03443-7

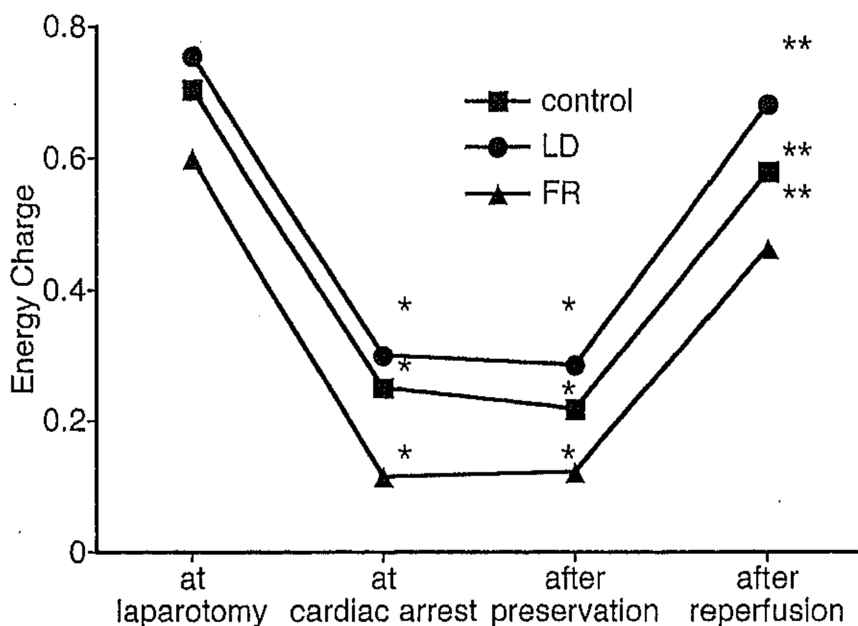


Fig 1. Changes in energy charge of the liver grafts: At laparotomy; at laparotomy of the donors; at cardiac arrest; 7 minutes after cardiac arrest; after preservation; after 3 hours of cold preservation; after reperfusion; 1 hour after reperfusion. *$P < .01$ vs at laparotomy; **$P < .01$ vs at cardiac arrest and after preservation; no significant change at cardiac arrest vs after preservation.

PGE_1 is known to have cytoprotective effects on ischemic and reperfusion injury and to improve the microcirculation of the liver.[2,3] FR is a dual inhibitor of TNF-α and IL-1β production.[4] Ulinastatin (UTI), protease inhibitor, hampers neutrophil elastase. We previously reported that the production of IL-1β in the graft from an NHBD was significantly elevated and could be suppressed by eliminating Kupffer cells.[5] We also reported that TNF-α induces superoxide generation by Kupffer cells, which mediates neutrophil accumulation and causes endothelial cell disruption.[6] In this study, we treated pigs with PGE_1, FR, and UTI, instead of Kupffer cell elimination. As electron microphotographs show, SLCs in the FR group were well preserved even after reperfusion, while sinusoidal structures were completely destroyed in the control group after reperfusion. Furthermore, in the FR group, the values of energy charge recovered, and all recipients survived longer than 7 days.

In summary, triple-drug therapy, using PGE_1, FR, and UTI, will prevent ischemia and reperfusion injuries in a liver graft, which will lead to safer LTx from NHBD.

Table 1. Recipient Survival After LTx

Group	Survival	Cause of Death
Control group	<12 h	Primary graft failure
(n = 5)	<12 h	Primary graft failure
	<12 h	Primary graft failure
	5 days	Graft failure
	5 days	Graft failure
LD group	<12 h	Pulmonary infarction
(n = 5)	>7 days	
	>7 days	
	>7 days	
	>7 days	
FR group	>7 days	
(n = 4)	>7 days	
	>7 days	
	>7 days	

REFERENCES

1. Tsukamoto S, Ohkohchi N, Orii T, et al: Transplantation 67:1396, 1999
2. Sugawara Y, Kubota K, Ogura T, et al: J Hepatol 29:969, 1998
3. Kishimoto S, Sakon M, Umeshita K, et al: Transplantation 69:2314, 2000
4. Yamamoto N, Sasaki F, Yamazaki H, et al: Eur J Pharmacol 314:137, 1996
5. Oikawa K, Ohkohchi N, Sato M, et al: Transpl Int 13:s573, 2000
6. Shibuya H, Ohkohchi N, Tsukamoto S, et al: Hepatology 26:113, 1997

Transpl Int (2003) 16: 100–107
DOI 10.1007/s00147-002-0498-6

ORIGINAL ARTICLE

Masahide Sato
Nobuhiro Ohkohchi
Shigeki Tsukamoto
Nozomi Koyamada
Takeshi Asakura
Yoshitaka Enomoto
Masahiro Usuda
Shigehito Miyagi
Akiho Okada
Susumu Satomi

Successful liver transplantation from agonal non-heart-beating donors in pigs

Received: 8 October 2001
Revised: 17 July 2002
Accepted: 22 August 2002
Published online: 19 December 2002
© Springer-Verlag 2002

M. Sato (✉) · S. Tsukamoto
N. Koyamada · T. Asakura
Y. Enomoto · M. Usuda · S. Miyagi
A. Okada · S. Satomi
Division of Advanced Surgical Science and Technology, Graduate School of Medicine, Tohoku University, 1-1 Seiryo-machi, Aobaku, Sendai, 980-8574 Japan
e-mail: m6sato@blue.ocn.ne.jp
Tel.: +81-22-7177214
Fax: +81-22-7177217

N. Ohkohchi
Department of Surgery, Institute of Clinical Medicine, University of Tsukuba, Tennoudai 1-1-1, Tsukuba, Japan

Abstract An effective way to overcome shortage of donors in liver transplantation (LTx) is to consider such from non-heart-beating donors (NHBDs). We investigated how a liver graft should be treated before and/or after procurement for successful LTx from an NHBD. Porcine LTx was performed with FR167653 (FR), a dual inhibitor of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and/or prostaglandin E_1 (PG). Animals were allocated to an FR group ($n=4$, donors and recipients were treated with FR), a PG group ($n=4$, donors and recipients were treated with PG), or an FRPG group ($n=4$, donors and recipients were treated with both FR and PG). No recipients in the FR group and only two of four recipients in the PG group survived, whereas all recipients in the FRPG group survived. Suppression of TNF-α and IL-1β and maintenance of microcirculation are the key to successful transplantation from NHBDs.

Keywords *Liver transplantation* Non-heart-beating donor · Warm ischemic injury · Reperfusion injury

Introduction

The shortage of donors is a serious problem in liver transplantation (LTx) all over the world. It has generally been accepted that liver grafts could only be transplanted from heart-beating donors (HBDs). However, if it were possible to transplant liver grafts from non-heart-beating donors (NHBDs), the number of LTxs would increase remarkably. Clinically, LTx from controlled NHBDs is being attempted in some institutions [3, 5, 16], but it has also been reported that the grafts from NHBDs failed due to primary graft non-function more often than those from HBDs. In previous studies [6, 20] we showed that microcirculatory disturbance after reperfusion caused primary graft non-function in liver grafts from NHBDs. Microcirculatory disturbance is caused by the deterioration of sinusoidal endothelial cells (SECs) and sinusoidal narrowing. SEC deterioration can be prevented by the elimination of Kupffer cells and sinusoidal narrowing with nafamostat mesylate (NM) rinse [21]. However, in clinical practice it seems difficult to transplant liver grafts in which Kupffer cells have been eliminated. On the other hand, we also reported that tumor necrosis factor-α (TNF-α) induced the generation of superoxide by Kupffer cells, and this led to mediate neutrophil accumulation [18]. Thus, in our present study, we performed porcine orthotopic LTx of liver grafts retrieved from agonal NHBDs using FR167653 (FR), a dual inhibitor of TNF-α and interleukin-1β (IL-1β) production, to control the chain reactions after reperfusion mentioned above, instead of eliminating Kupffer cells. The aim of this study

was to establish a method leading to successful LTx from agonal NHBDs in clinical practice.

Materials and methods

All experiments were conducted according to the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health.

Experimental design

White Landrace pigs, weighing 20–30 kg, were allocated to five groups as follows:

1. Control group ($n=4$): donor and recipient pigs received no treatment. Immediately before anastomosis of the portal vein, the liver grafts were rinsed with 400 ml Ringer's lactate.
2. FR group ($n=4$): 1.0 mg/kg/h of FR167653 (FR; Fujisawa, Tokyo, Japan), a dual inhibitor of TNF-α and IL-1β production [25], was infused continuously during both donor and recipient surgery. The liver grafts were rinsed with 400 ml NM rinse (Table 1; Torii, Tokyo, Japan).
3. PG group ($n=4$): 20 ng/kg/min of prostaglandin E_1 (PG; Ono, Tokyo, Japan) was administered intravenously during donor and recipient surgery. The liver grafts were rinsed with NM rinse immediately before anastomosis of the portal vein.
4. FRPG group ($n=4$): donor and recipient pigs were treated with both FR and PG in the same manner as above. The liver grafts were rinsed with NM rinse.
5. HBD group ($n=4$): donor and recipient pigs did not receive any treatment, and the liver grafts were rinsed with NM rinse. The rinsing solution was kept at approximately 4 °C. LTx was performed after the animals had been starved for 24 h, during which time they were allowed to drink water ad libitum.

Donor operation

After the pigs had been pre-medicated with atropine injected intramuscularly, anesthesia was maintained with an isoflurane/oxygen mixture and pancuronium bromide. A catheter was placed in the external jugular vein to monitor central vein pressure, and another was placed in the common carotid artery to monitor blood pressure. After laparotomy had been performed, 10 mg/kg methylprednisolone and 30,000 U/kg ulinastatin (UTI; Mochida, Tokyo, Japan) were administered intravenously. The liver was retrieved as in humans, with minor modifications. First, the splenic vein and common bile duct were cannulated after heparin (300 U/kg) had been administered intravenously. We cannulated the abdominal aorta and stopped the donor's respiration by using pancuronium bromide and detaching the respirator. Central vein pressure was measured and kept below 14 cm H_2O until cardiac arrest was identified on the electrocardiogram. Ten minutes after cardiac arrest, perfusion of the liver with University of Wisconsin (UW) solution was started. UW solution (500 ml) was infused through the portal vein and the abdominal aorta, after which the liver was retrieved. The liver was promptly flushed with 50 ml UW solution through the hepatic artery and was stored in two plastic bags in an ice-slush bath for 3 h. In the HBD group, the liver was procured as mentioned above, but not in the agonal state.

Table 1 Composition of NM rinse

Parameter	Value
Na-lactobionate	110 mmol/l
NaH_2PO	25 mmol/l
Raffinose	30 mmol/l
Allopurinol	1 mmol/l
Glutathione	3 mmol/l
NM	0.8 mmol/l
pH	7.44
Osmotic pressure	290–300 mosmol/l

Recipient operation

The recipient pigs were anesthetized in the same way as the donors were. The right carotid artery and external jugular vein were cannulated for monitoring of blood pressure and for blood sampling and intravenous fluid infusion, respectively. After laparotomy, the liver was dissected free and removed. During the anhepatic phase, the blood of the portal vein and infra-hepatic inferior vena cava was shunted to the left external jugular vein with a biopump (Medtronic Bio-Medicus, Eden Prairie, Minn., USA). As anticoagulant, NM was infused continuously at 1 mg/kg/h until the biopump was stopped. The liver graft was implanted orthotopically with end-to-end anastomosis of the supra-hepatic inferior vena cava, the portal vein, the infra-hepatic inferior vena cava (all with 4-0 Prolene, running suture), and the hepatic artery (8-0 Prolene, intermittent suture) in that order. The liver graft was reperfused with portal blood after the portal vein anastomosis had been completed. The bile duct was drained via an external tube fistula. FK506 was administered postoperatively at 0.1 mg/kg/day to recipient pigs for immunosuppression.

Examination items

Tissue specimens were taken from the edges of each liver graft at laparotomy, 7 min after cardiac arrest, and 1 h after reperfusion.

Energy charge of liver tissue

To measure energy charge, we took tissue specimens, using tongs, pre-cooled the specimens in liquid nitrogen, and then stored them at −80 °C. After extraction with 0.6 N perchloric acid, adenosine triphosphate, diphosphate, and monophosphate were measured by high-performance liquid chromatography (Jasco HPLC Analyzer System; Nihon Bunko, Tokyo, Japan). The eluent was 60-mM phosphate buffer (pH 6.0), and the flow rate was 1.0 ml/min at room temperature. The energy charge was calculated according to the formula proposed by Atkinson and Chapman [1].

Proton ATPase activity of liver tissue

Proton ATPase activity was determined as described by Seya et al. [17]. Briefly, mitochondrial pellets were suspended in a potassium buffer solution, and Dis3C, a fluorescent dye, was added. After the electron transport chain had been stopped by the addition of 250 ng of antimycin A, changes in the mitochondrial membrane potential upon the addition of 2 μmol of ATP to the mixture were measured by fluorometry (Jasco FP-777; Nihon Bunko) at 23 °C, at an excitation wavelength of 625 nm and an emission wavelength of 670 nm.

Histological examination

Tissue specimens for microscopy were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin wax. Sections 5 μm thick were stained with hematoxylin and eosin. Tissue specimens for electron microscopy were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). After treatment with 1% osmium tetroxide, they

were dehydrated through a graded series of ethanol and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate, and zone 1 of the liver acini was examined under an electron microscope (JEM-1010; Japan Electron Optics Laboratories, Tokyo, Japan). The reasons we selected zone 1 for examination were that injuries of SECs are more prominent in the early period of cold preservation [11] and that more severe injuries may occur due to Kupffer cells at reperfusion.

Aspartate aminotransferase and lactate dehydrogenase

Serum aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured with commercially available kits (AST: Iatron, Tokyo, Japan; LDH: Wako Pure Chemical Industries, Osaka, Japan).

Statistics

All values are presented as mean ± SD. We analyzed the differences among the five groups, using one-factor ANOVA, and then made pairwise comparisons using a post-hoc test. *P* values of below 0.05 were considered statistically significant.

Results

Recipient survival

The mean duration of warm ischemia from the time the respirator was turned off until cardiac arrest was 31.2±2.9 min, and there were no statistical differences between the groups except for the HBD group. The mean time of cold ischemia was 3.0±0.3 h, and there were no statistical differences between the five groups. In the control and the FR groups, no recipient survived for longer than 24 h. Massive hemorrhagic ascites were found at autopsy. In the PG group, two recipients died within 24 h, and findings similar to those observed in the control and FR groups were found at autopsy. Two of four recipients survived for more than 7 days. In the FRPG and HBD groups, all four recipients survived for more than 7 days (Table 2).

Serum AST and LDH

Serum AST and LDH increased after reperfusion in all but the HBD group and especially in the FR group. On postoperative day 1, AST and LDH remained high and then decreased. AST levels normalized by postoperative day 7. There were no statistical differences between the groups in each phase (Fig. 1).

Energy charge of liver tissue

The energy charge at laparotomy was 0.601 ±0.134. The values decreased significantly to less than 50% of the initial value in all but the HBD group at 7 min after cardiac arrest. There were no statistical differences between the four NHBD groups. One hour after reperfusion, the value recovered to 50–85% of that at laparotomy in the FR, PG, and FRPG groups, but did

Table 2 Recipient survival after LTx

Group	Survival	Cause of death
Control group (*n*=4)	<24 h	Primary graft failure
	<24 h	Primary graft failure
	<24 h	Primary graft failure
	<24 h	Primary graft failure
FR group (*n*=4)	<24 h	Primary graft failure
	<24 h	Primary graft failure
	<24 h	Primary graft failure
	<24 h	Primary graft failure
PG group (*n*=4)	<24 h	Primary graft failure
	<24 h	Primary graft failure
	>7 Days	Killed
	>7 Days	Killed
FRPG group (*n*=4)	>7 Days	Killed
	>7 Days	Killed
	>7 Days	Killed
	>7 Days	Killed
HBD group (*n*=4)	>7 Days	Killed
	>7 Days	Killed
	>7 Days	Killed
	>7 Days	Killed

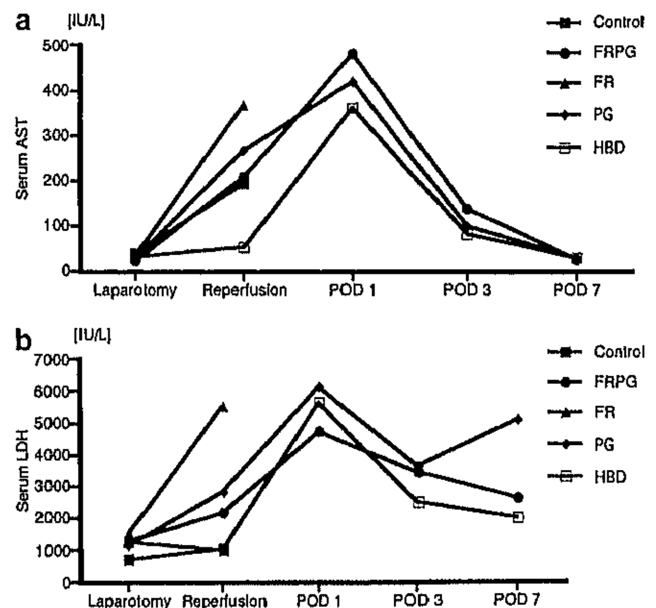


Fig. 1 Serum AST level (**a**) and serum LDH level (**b**). Both AST and LDH increased after reperfusion in all but the HBD group, and especially in the FR group. On postoperative day 1, AST and LDH remained high and then decreased. AST levels normalized by postoperative day 7. There were no statistical differences between the groups in each phase. (*Laparotomy* at laparotomy of the recipients, *Reperfusion* 5 min after reperfusion, *POD* postoperative day)

not recover in the control group. In the FR group, the recovery was less than in the PG and FRPG groups. There were no statistical differences between the five groups in the values 1 h after reperfusion (Fig. 2).

Mitochondrial proton ATPase activity in the liver graft

Proton ATPase activity at laparotomy was 55.78± 11.32%. ATPase activity decreased 7 min after cardiac arrest in the control, FR, and PG groups, but was maintained in the FRPG group. One hour after reperfusion, the values decreased remarkably in the control, FR, and PG groups. On the other hand, the value was maintained at the same level as that observed at laparotomy in the FRPG and HBD groups, and was significantly higher than those in the other groups (Fig. 3).

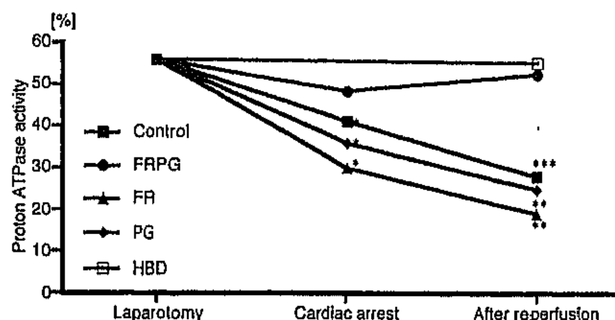


Fig. 3 Changes in proton ATPase activity of the liver grafts. The ATPase activity decreased after cardiac arrest in the control, FR, and PG groups, but was maintained in the FRPG group. After reperfusion, the values decreased remarkably in the control, FR, and PG groups. On the other hand, the value was maintained at the same level as that observed at laparotomy in the FRPG and HBD groups, and they were significantly higher than those in the other groups. $*P<0.05$ vs FRPG, $**P<0.05$ vs FRPG and HBD, $***P<0.05$ vs HBD (*Laparotomy* at laparotomy of the donors, *Cardiac arrest* 7 min after cardiac arrest, *After reperfusion* 1 h after reperfusion)

Microscopy findings

At 7 min after cardiac arrest, the sinusoidal spaces in zone 1 were wide in the PG and FRPG groups, but in the control and FR groups the sinusoidal spaces were narrow (Fig. 4). One hour after reperfusion, the sinusoidal spaces were narrow, and massive bleeding was recognized in the control, FR, and PG groups. Partial necrosis of hepatocytes in zone 2 was observed in the three groups. These changes were not observed in the FRPG and HBD groups (Fig. 5).

Electron microscopy findings

At 7 min after cardiac arrest, both SECs and hepatocytes were almost normal except for hypoxic vacuoles in the PG and FRPG groups, but deterioration of SECs was recognized and spillage of the cytoplasm into the sinusoidal space observed in the control and FR groups. Hypoxic vacuoles in hepatocytes were also observed in these two groups (Fig. 6). One hour after reperfusion, both SECs and hepatocytes were observed to be well preserved in the FRPG and HBD groups. SECs and Disse's space were completely destroyed in the control, FR, and PG groups and hepatocyte microvilli were hardly recognized. Spillage of the cytoplasm into the sinusoidal space was observed, and erythrocytes were found in Disse's space (Fig. 7).

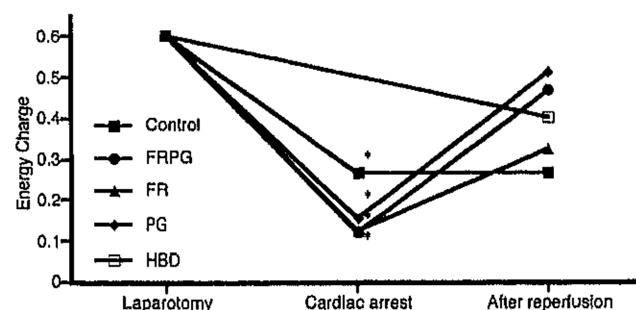


Fig. 2 Changes in energy charge of the liver grafts. After cardiac arrest, the energy charge values of non-HBD groups significantly decreased and then recovered after reperfusion, except for the control group. There were no statistical differences between the values in all groups at 1 h after reperfusion. $*P<0.05$ vs initial value (*Laparotomy* at laparotomy of the donors, *Cardiac arrest* 7 min after cardiac arrest, *After reperfusion* 1 h after reperfusion)

Discussion

The number of patients on waiting lists for LTx has been increasing, and the waiting time has become longer [2]. Surgeons are forced to consider organs from suboptimal donors, such as NHBDs, and some trials are underway at some institutions. However, LTx from NHBDs is seldom performed, since their liver grafts are very susceptible to primary graft non-function. We have previously reported that microcirculatory disturbance was a main obstacle to successful LTx from NHBDs and that this disturbance was due to two major factors: SEC deterioration and sinusoidal narrowing. Furthermore, we also reported that elimination of Kupffer cells prevented SEC deterioration and that sinusoidal narrowing was prevented by NM rinse [22]. In that study, Kupffer cells were eliminated by liposome-encapsulated dichloromethylene diphosphonate (L-DMDP). However, to eliminate Kupffer cells, L-DMDP had to be administered 42 h before the procurement of liver grafts [22]. It seems difficult to apply this procedure to clinical practice. Therefore, a new strategy to control Kupffer-

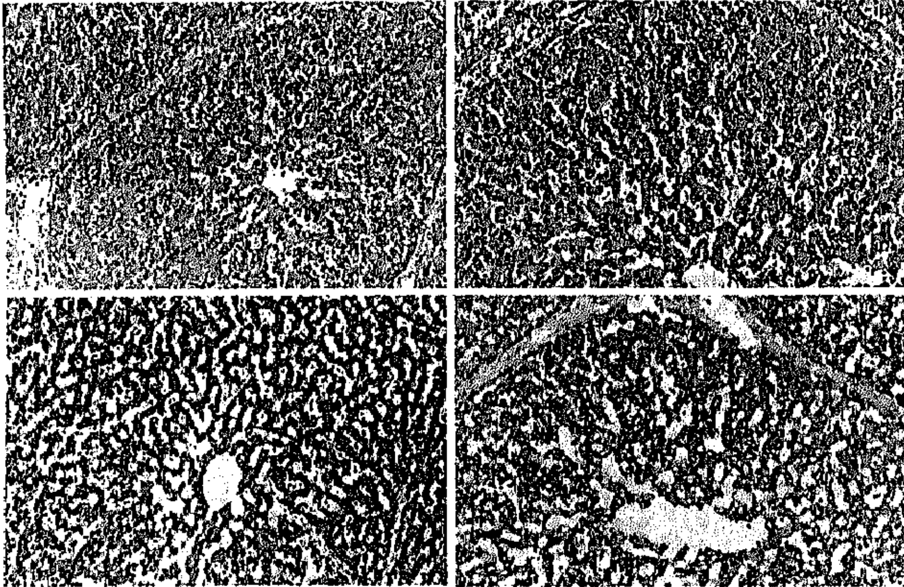


Fig. 4 Microphotograph of the livers 7 min after cardiac arrest (hematoxylin and eosin stain, original magnification ×40 on 35-mm film). The sinusoidal spaces in zone 1 were wide in the PG and FRPG groups, but in the control and the FR groups, the sinusoidal spaces were narrow

cell function should be considered. In the present study, we clearly demonstrated that FR, which is a dual inhibitor of TNF-α and IL-1β production, prevents SEC deterioration at reperfusion in LTx from NHBDs.

Previously, we demonstrated that suppression of Kupffer-cell function was an important key to successful LTx from agonal NHBDs. In agonal NHBDs, cytokines liberated by the gut prime the liver graft, and especially Kupffer cells. The primed Kupffer cells are stimulated and excited during cold ischemia [13]. We previously reported that TNF-α induced the generation of superoxide by Kupffer cells, and this led to mediate neutrophil accumulation [18]. Neutrophil accumulation is probably associated with microvascular damage [7], and adherent neutrophils can secrete numerous mediators including reactive oxygen species, cytokines, and proteases, greatly amplifying the inflammatory response. Our previous

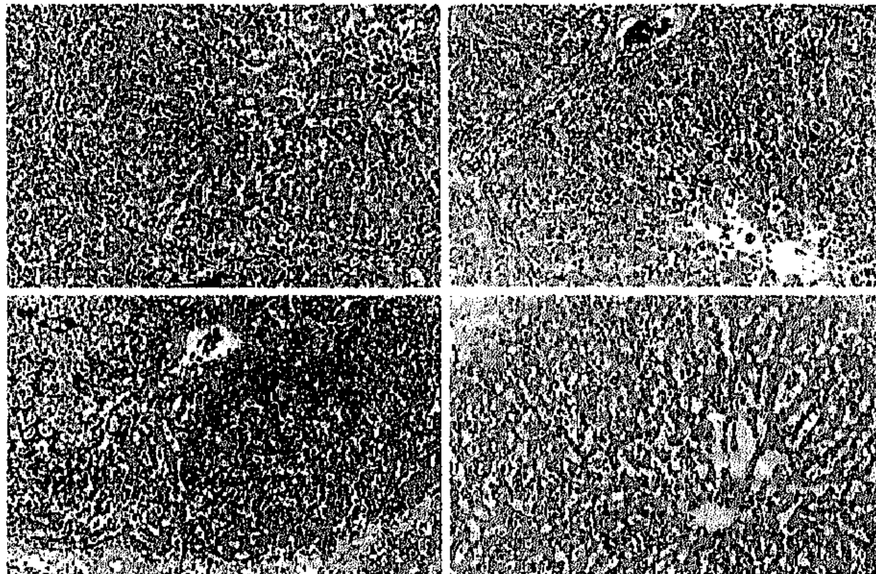
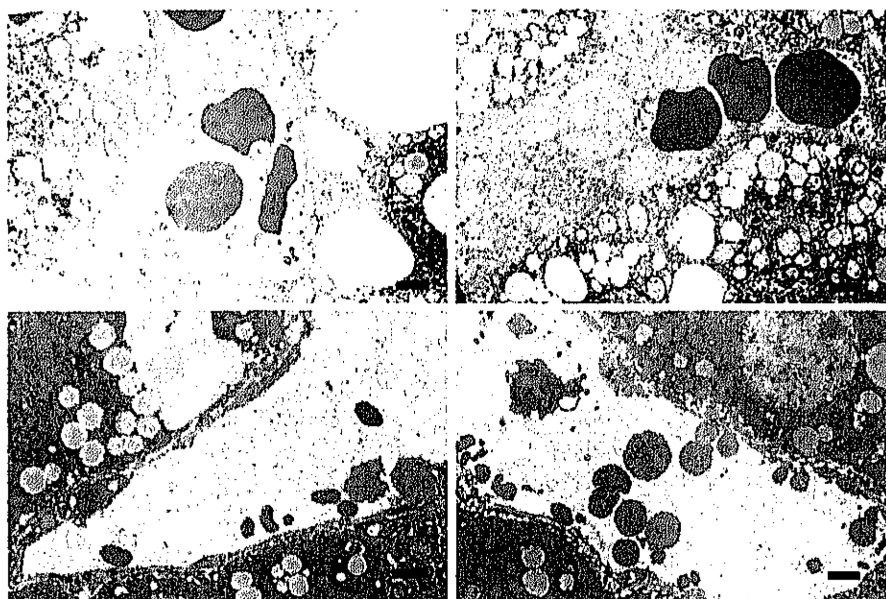


Fig. 5 Microphotograph of the livers 1 h after reperfusion (hematoxylin and eosin stain, original magnification ×40 on 35-mm film). The sinusoidal spaces were narrow and massive bleeding was recognized in the control, FR, and PG groups. Partial necrosis of hepatocytes in zone 2 was observed in the three groups. These changes were not observed in the FRPG and the HBD groups

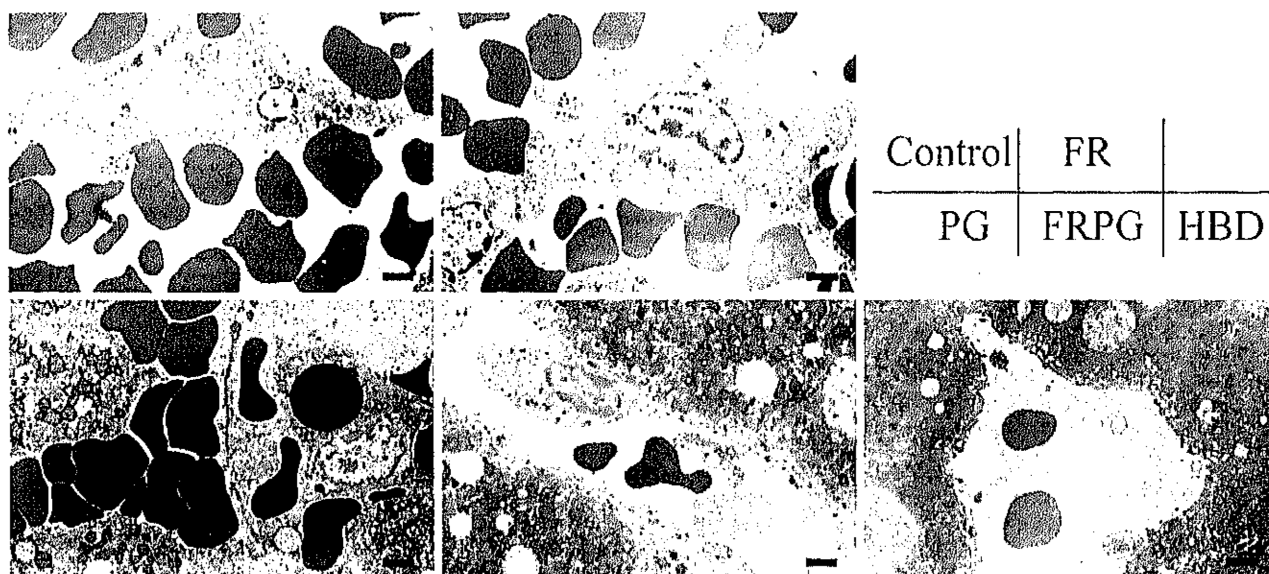


Control	FR
PG	FRPG

Fig. 6 Electron microphotograph of the livers 7 min after cardiac arrest. SECs were almost normal in the PG and FRPG groups, but deterioration of SECs was recognized and spillage of the cytoplasm into the sinusoidal space was observed in the control and the FR groups. *Scale bar* = 2 μm

studies suggested that the inhibition of TNF-α production would suppress Kupffer cell activation and prevent the chain reaction following reperfusion injury, as mentioned above [18]. We have also reported that the production of IL-1β was significantly high in liver grafts from NHBDs [14]. Thus, in our present study we administered FR to control the chain reactions mentioned above instead of eliminating Kupffer cells. In this study, electron microscopy after reperfusion revealed distinct disruption and detachment of SECs in liver grafts in the PG group, whereas in those in the FRPG group the injury was much milder. These results indicate that inhibition of TNF-α production to suppress Kupffer-cell function as mentioned above plays an important role in SEC deterioration after LTx from agonal NHBDs.

Fig. 7 Electron microphotograph of the livers 1 h after reperfusion. Both SECs and hepatocytes were observed to be well preserved in the FRPG and HBD groups. SECs and Disse's space were completely destroyed in the control, FR, and PG groups, and hepatocyte microvilli were hardly recognizable. Spillage of the cytoplasm into the sinusoidal space was observed and erythrocytes were found in Disse's space. *Scale bar* = 2 μm



The question of difference between liver grafts from HBDs and those from NHBDs is whether the liver grafts suffer warm ischemia or not. The viability of liver grafts from NHBDs is impaired by warm ischemia before procurement. Takada et al. reported that after 60 min of warm ischemia with no pre-treatment, liver grafts were still suitable for transplantation [19]. In this study, the donors were subjected to approximately 30 min of warm ischemia; nevertheless, the survival in the control group was worse than that reported by Takada et al. This may have been due to our NHBD model. In contrast to our model, the NHBD models used in the experimental studies conducted so far rarely pertain to the agonal state. In an as yet unpublished study we have reported that the cytokines liberated from the gut immediately before death primed the liver graft for an exaggerated response to reperfusion through cold ischemia (Miyagi et al., Role of arachidonic acid cascade in warm ischemia-reperfusion injury of liver grafts from non-heart-beating rats, manuscript submitted). It was also reported that a large increase in cytosolic free calcium concentration was an important initial step in the sequence of events leading to cell damage during warm ischemia [4, 8]. PG was reported to delay the rise in cytosolic free calcium concentration and to prevent plasma membrane bleb formation [10]. In this study, electron microscopy at retrieval revealed distinct disruption of SECs in liver grafts in which PG was not administered, whereas in those in which PG was administered the injury was much milder. Our results suggest that SECs deteriorated immediately before death and that it is important for successful LTx from NHBDs that precautionary measures against this be taken.

NM and UTI are serine protease inhibitors. Protease inhibitors prevent ischemia-reperfusion injury, but the underlying mechanisms for this action remain obscure. Our previous study indicated that NM rinse induced the widening of hepatic sinusoids and protected SECs to some degree; however, the protective effect of NM was insufficient to prevent the chain reaction elicited by reperfusion injury which starts with the activation of Kupffer cells [18]. In our present study, microscopy revealed that all grafts except those in the control group were reperfused well. Furthermore, we have reported that NM regulates arachidonate metabolites, especially thromboxaneA2 (TXA2) (Miyagi et al., manuscript submitted). Inhibition of TXA2 synthesis suppresses the liberation of other vasoconstrictive substances and neutrophil infiltration, thereby improving microcirculation [12, 26]. UTI, which is present in human urine, has two cardinal actions: (1) the suppression of neutrophil elastase (NE) and cathepsin G and (2) the stabilization of lysosomal and cellular membranes [9, 15, 22, 23]. It was also reported that UTI reduced the production of cytokine-induced neutrophil chemoattractant by Kupffer cells stimulated with NE, attenuating ischemia-reperfusion injury of the liver [24]. In this study, UTI prevented the development of acidosis after reperfusion in the FRPG group (data not shown). Our results suggest that NM rinse and UTI were effective in the protection against ischemia-reperfusion injury, although further examination is needed.

In summary, our results indicate that PG saved SECs from warm ischemic damage and that FR prevented SEC deterioration at reperfusion. The combination therapy, consisting of PG, FR, and protease inhibitors, may have a role in clinical LTx from NHBDs.

References

1. Atkinson DE, Chapman AG (1979) The adenylate energy charge in the study of enzymes in vitro. Methods Enzymol 55:229–235
2. Bismuth H (1994) The need for a consensus agreement on indications of liver transplantation. Hepatology 20:1S–2S
3. Casavilla A, Ramirez C, Shapiro R, Nghiem D, Miracle K, Bronsther O, Randhawa P, Broznick B, Fung JJ, Starzl T (1995) Experience with liver and kidney allografts from non-heart-beating donors. Transplantation 59:197–203
4. Clavien P, Harvey P, Strasberg S (1992) Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. Transplantation 53:957–978
5. D'Alessandro AM, Hoffmann RM, Knechtle SJ, Eckhoff DE, Love RB, Kalayoglu M, Sollinger HW, Belzer FO (1995) Successful extrarenal transplantation from non-heart-beating donors. Transplantation 59:977–982
6. Endoh T, Ohkohchi N, Katoh H, Seya K, Satomi S, Mori S, Nakamura K (1996) Graft conditioning of liver in non-heart-beating donors by an artificial heart and lung machine in situ. Transplant Proc 28:110–115
7. Ferguson D, McDonagh PF, Biewer J, Paidas CN, Clemens MG (1993) Spatial relationship between leukocyte accumulation and microvascular injury during reperfusion following hepatic ischemia. Int J Microcirc Clin Exp 12:45–60
8. Gasbarrini A, Borle AB, Farghali H, Bender C, Francavilla A, van Thiel D (1992) Effect of anoxia on intracellular ATP, Na+i, Ca2+i, Mg2+i, and cytotoxicity in rat hepatocytes. J Biol Chem 267:6654–6663
9. Jaeschke H, Farhood A, Smith CW (1990) Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. FASEB J 4:3355–3359
10. Kishimoto S, Sakon M, Umeshita K, Miyoshi H, Taniguchi K, Meng W, Nagano H, Dono K, Ariyosi H, Nakamori S, Kawasaki T, Gotoh M, Monden M, Imajoh-Ohmi S (2000) The inhibitory effect of prostaglandin E_1 on oxidative stress-induced hepatocyte injury evaluated by calpain-μ activation. Transplantation 69:2314–2319

11. Koizumi M, Ohkohchi N, Katoh H, Koyamada N, Fujimori K, Sakurada M, Andoh T, Satomi S, Sasaki T, Taguchi Y, Mori S, Kataoka S, Yamamoto TY (1989) Preservation and reflow damage in liver transplantation in the pig. Transplant Proc 21:1323–1326
12. Kurihara T, Akimoto M, Kurokawa K, Ishiguro H, Niimi A, Maeda A, Sigemoto M, Yamashita K, Yokoyama I, Hirayama Y, Ihara M, Yano M (1992) Relationship between endothelin and thromboxane A2 in rat liver microcirculation. Life Sci 51:PL281–PL285
13. Mochida S, Arai M, Ohno A, Masaki N, Ogata I, Fujiwara K (1994) Oxidative stress in hepatocytes and stimulatory state of Kupffer cells after reperfusion differ between warm and cold ischemia in rats. Liver 14:234–240
14. Oikawa K, Ohkohchi N, Sato M, Satomi S (2000) The effects of the elimination of Kupffer cells in the isolated perfused liver from non-heart-beating rats. Transpl Int 13 [Suppl 1]:S573–S579
15. Poggetti RS, Moore FA, Moore EE, Bensard DD, Anderson BO, Banerjee A (1992) Liver injury is a reversible neutrophil-mediated event following gut ischemia. Arch Surg 127:175–179
16. Reich DJ, Munoz SJ, Rothstein KD, Nathan HM, Edwards JM, Hasz RD, Manzarbeitia CY (2000) Controlled non-heart-beating donor liver transplantation: a successful single-center experience, with topic update. Transplantation 70:1159–1166
17. Seya K, Ohkohchi N, Watanabe N, Shibuya H, Taguchi Y, Mori S (1994) Rapid fluorometric assay for mitochondrial proton adenosine triphosphatase activity for assessment of viability of liver graft tissue. J Clin Lab Anal 8:418–423
18. Shibuya H, Ohkohchi N, Tsukamoto S, Satomi S (1997) Tumor necrosis factor-induced, superoxide-mediated neutrophil accumulation in cold ischemic/reperfused rat liver. Hepatology 26:113–120
19. Takada Y, Taniguchi H, Fukunaga K, Yuzawa K, Otsuka M, Todoroki T, Iijima T, Fukao K (1997) Hepatic allograft procurement from non-heart-beating donors: limits of warm ischemia in porcine liver transplantation. Transplantation 63:369–373
20. Tsukamoto S, Ohkohchi N, Endoh T, Seya K, Satomi S, Mori S (1996) Procurement of liver grafts by an artificial heart-lung machine using leukocyte-depleted washed red blood cells in non-heart-beating donors. Transplant Proc 28:197–200
21. Tsukamoto S, Ohkohchi N, Fukumori T, Orii T, Asakura T, Takayama J, Shibuya H, Katoh H, Satomi S (1999) Elimination of Kupffer cells and nafamostat mesylate rinse prevent reperfusion injury in liver grafts from agonal non-heart-beating donors. Transplantation 67:1396–1403
22. Watanabe K, Konishi K, Fujioka M, Kinosita S, Nakagawa H (1989) The neutrophil chemoattractant produced by the kidney epithelial cell line NRK-52E is a protein related to the KC/gro protein. J Biol Chem 264:19559–19563
23. Watanabe K, Suematsu M, Iida M, Takahashi K, Iizuka Y, Suzuki H, Suzuki M, Tsuchiya M, Tsurufuji S (1992) Effect of rat CINC/gro, a member of the interleukin-8 family, on leukocytes in microcirculation of the rat mesentery. Exp Mol Pathol 56:60–69
24. Yamaguchi Y, Ohshiro H, Nagao Y, Odawara K, Okabe K, Hidaka H, Ishihara K, Uchino S, Furuhashi T, Yamada S, Mori K, Ogawa M (2000) Urinary trypsin inhibitor reduces C-X-C chemokine production in rat liver ischemia/reperfusion. J Surg Res 94:107–115
25. Yamamoto N, Sakai F, Yamazaki H, Nakahara K, Okuhara M (1996) Effect of FR167653, a cytokine suppressive agent, on endotoxin-induced disseminated intravascular coagulation. Eur J Pharmacol 314:137–142
26. Yokoyama I, Kobayashi T, Negita M, Hayashi S, Yasutomi M, Katayama A, Uchida K, Takagi H (1996) Liberation of vasoactive substances and its prevention with thromboxaneA2 synthase inhibitor in pig liver transplantation. Transpl Int 9:76–81

ELSEVIER

Mechanisms of Preservation and Ischemic/Reperfusion Injury in Liver Transplantation

N. Ohkohchi

IN ORGAN TRANSPLANTATION, the graft suffers from warm ischemic injury due to the donor's physical condition, ie, hypotension and hypoxia, prior to the retrieval. When the organ is retrieved, it receives additional warm ischemic injury for approximately 10 minutes after the aorta is clamped until the graft is cooled down. The graft also receives cold ischemic injury during preservation. After reperfusion following cold ischemia, the graft receives further injury. Previous studies of preservation and reperfusion injury of the liver showed that hepatocytes were damaged due to warm ischemia, and sinusoidal endothelial cells were damaged due to cold ischemia. However, recent studies about warm ischemia/reperfusion injury[1] and about liver transplants from donors after cardiac arrest[2] indicate that sinusoidal endothelial cells are damaged before hepatocytes, both during warm and cold ischemia. Therefore, preventing damage to sinusoidal endothelial cells to prevent narrowing and maintain the microcirculation following reperfusion is important in liver transplantation. Addressing these injuries is important to prevent primary graft non-function, in other words, to maintain good graft function. In this article, we review recent findings on ischemia/reperfusion injury in liver transplantation and discuss the drugs that are effective for prevention of microcirculatory disturbance.

COLD PRESERVATION AND DETERIORATION OF SINUSOIDAL ENDOTHELIAL CELLS

The precise mechanisms in the liver following cold preservation are unclear. According to a previous report,[3] when rat livers are simply stored in a solution containing 0.9% NaCl and 2 mmol/LCaCl2, sinusoidal endothelial cells disappear almost completely within 8 hours, an irreversible change. In contrast, the bleb formation observed in hepatocytes is a reversible change. It is not clear whether morphological changes in hepatocytes are reversible up to 24 hours of cold storage; this report concluded that sinusoidal endothelial cells are susceptible to primary injury during cold preservation. Changes in sinusoidal endothelial cells occurring during several hours of cold preservation in Euro-Collins solution (EC solution) have been observed with a transmission electron microscope, including formation of secondary lysosome. Swelling of the sinusoidal endothelial cells and degeneration of the cell membranes have also been observed in time-dependent fashion. However, when the University of Wisconsin solution (UW solution) is used for the preservation solution, the damages are suppressed.[4] Although the reasons for the effectiveness of UW solution have not been completely clarified, large-molecule components of the UW solution probably contribute to the maintenance of the sinusoidal structure.

With regard to the ionic components of the solution, some studies reported a similar preservation efficacy using an extracellular fluid-based solution as the UW solution.[5] On the other hand, there are some reports that preservation solutions with low K^+ concentrations are better.[6] Thus, it has not been agreed which components of the preservation solution are necessary.

Recently it has been reported that apoptosis mediates the deterioration in sinusoidal endothelial cells during cold preservation.[7] Calpain inhibitors seem to be effective in the prevention of apoptosis of sinusoidal endothelial cells during cold preservation, because calpain a calcium-dependent enzyme, is related to the mechanism of apoptosis.[8] Other studies reported that vascular endothelial growth factor (VEGF) receptors, which are important in the maintenance and proliferation of these cells, are present on sinusoidal endothelial cells; indeed, VEGF seems to inhibit apoptosis during cold preservation.[9]

In addition to the changes in sinusoidal endothelial cells, Kupffer cell activation is another important target of injury during cold preservation of the liver. Morphologic studies show that Kupffer cells are activated during cold preservation, and this activation is inhibited more effectively by UW than by EC solution.[10,11] However, the level of activation is time-dependent.[12] Kupffer cell activation seems to be strongly related to the reperfusion injury.

REPERFUSION AND DETERIORATION OF SINUSOIDAL ENDOTHELIAL CELL

There is no doubt that Kupffer cells and neutrophils play a primary role in sinusoidal endothelial cell damage due to

From the Department of Surgery, Institute of Clinical Medicine, Tsukuba University, Tsukuba, Japan.

Address reprint requests to Ohkohchi Nobuhiro, MD, Department of Surgery, Institute of Clinical Medicine, Tsukuba University, Tennodai 1-1-1, Tsukuba, Japan.

0041-1345/02/$-see front matter
PII S0041-1345(02)03370-5

© 2002 by Elsevier Science Inc.
360 Park Avenue South, New York, NY 10010-1710

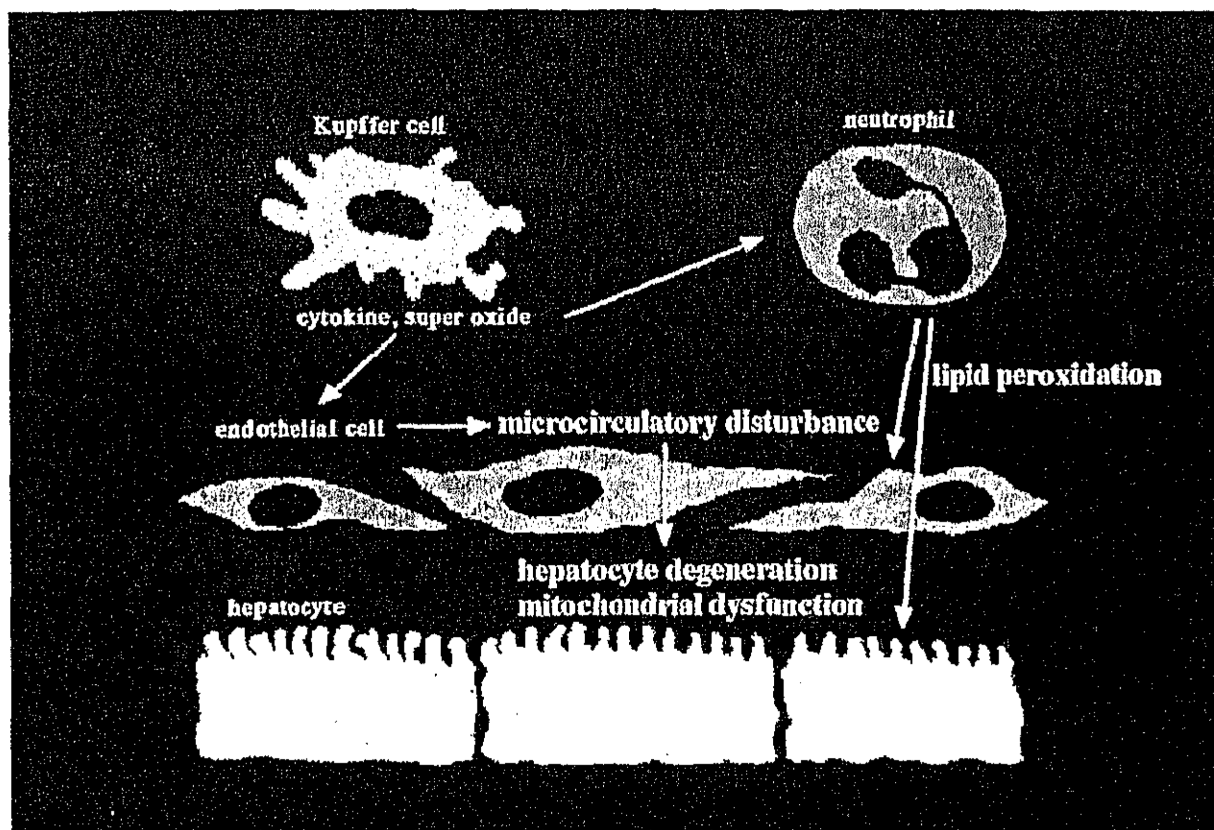


Fig 1. Schema of ischemia/reperfusion injury of the liver.

reperfusion. Kupffer cells activated during cold preservation release cytokines such as TNF-α and free radicals. The release of cytokines and radicals cause not only lipid peroxidation of the graft, but also the accumulation of neutrophils in the liver via these chemical mediators. The Kupffer cell activation results in the further generation of free radicals from neutrophils.[13,14] Sinusoidal endothelial cells that have been deteriorated during cold preservation sustain further damage by the large amount of free radicals that are released as described above from neutrophils. The increment in tissue factor by Kupffer cell activation causes disturbance of equilibrium of the intra-sinusoidal blood flow as well as by the free radical generation. As a result, intra- sinusoidal blood coagulation leads to a microcirculatory disturbance (Fig 1).

Kupffer cells play a role as initiators of the reperfusion injury to the liver graft, as mentioned above. Therefore, it is possible that various biological responses may be suppressed if Kupffer cell functions can be controlled. Gadolinium chloride and chlodronate liposomes have been used to kill or eliminate Kupffer cells in experimental studies. However, because they are considered potentially cytotoxic, they have not been used in clinical situations. Recently, it has been reported that glycine inhibits Kupffer cell function and reduces the reperfusion injury.[15] In an other report it was suggested that Kupffer cell activation may be inhibited through control of the Cl^- channel by glycine.[16] But the nature of the effect of glycine in the prevention of ischemia/reperfusion injury remains unclear. There is an interesting report that the method of retrieval of the graft influences Kupffer cell function.[17]

In addition to radicals, elastase in neutrophils is an important element in sinusoidal endothelial cell damage. Neutrophils release proteases and active oxygen radicals as a part of their biological self-protective function to break down proteins in foreign bodies, bacteria, and metabolites. Elastase, which is one of the proteases that is released from neutrophils as an active protease, causes severe tissue damage. Under normal circumstances, elastase activity is controlled by inhibitors that inactivate elastase. In addition to a large amount of elastase released in inflammatory lesions active oxygen and myeloperoxidase inactivate the inhibitors. As a result, elastase in the inflammatory lesion disrupts proteins, acting as a biological phylactic. However, in the ischemia/reperfusion injuries, the balance of elastase and its inhibitors is disturbed. Furthermore, elastase seems to lose the ability of self-recognition; tissue destruction becomes dominant. Studies in which the inhibitor for neutrophil elastase was administered in ischemia/reperfusion injury models indicate that sinusoidal endothelial cells

were effectively protected and neutrophils and adhesion to sinusoidal endothelia and invasion attenuated,[18] improving blood flow in the graft.[19] Inflammatory mediator production and the expression of nuclear factor-kβ (NF-kB) also was inhibited.[20] These reports suggest that elastase inhibitors are effective for prevention of ischemia/reperfusion injuries. In a report on adenosine, which is regarded to be an endogenous regulator of hepatic blood flow, an A (2A) receptor agonist attenuated the reperfusion injury, and an A (2A) receptor antagonist exacerbated the injury. These reports suggested that stimulation of A (2A) receptors is associated with inflammatory mediator production and neutrophil degranulation.[21]

In regard to intra-sinusoidal coagulation, the effectiveness of anti-coagulant therapy using anti-thrombin III has been reported.[22] However, coagulopathy is a major problem in the clinical apparatus of this treatment. The expression of thrombomodulin on endothelial cells in hepatic sinusoids is lower than that on the capillary endothelial cells in other organs. Moreover, the anticoagulant activity of thrombomodulin decreases following cold preservation. Since the level of anticoagulant activity in sinusoidal endothelial cells at the time of reperfusion is low, and the expression of tissue factor from Kupffer cells is increased following exposure to endotoxin derived from the intestine, the sinusoids show significantly increased coagulation activity. This imbalance between coagulation and anticoagulant activity produces intra-sinusoidal coagulation, suggesting that anticoagulants such as tissue factor pathway inhibitor (TFPI)[23,24] or recombinant thrombomodulin[25,26] are expected to maintain a balance of coagulation. However, the efficacy of these drugs in liver transplantation has not yet been reported.

REPERFUSION AND SINUSOIDAL NARROWING

In regard to the rinse solutions to wash out the UW perfusate from liver grafts prior to reperfusion, it has been reported that some rinse solutions alleviate sinusoidal narrowing or constriction, namely Carolina rinse.[2] Futhan rinse,[27] and a rinse containing an endothelin antagonist.[28] The characteristics of the Carolina rinse are several: its colloid osmotic pressure prevents cell swelling following wash out; it inhibits Kupffer cell function because it contains a calcium channel antagonist; it attenuates damage caused by active oxygen radicals because it contains an antioxidant; and it attenuates hypoxic cell damage that occurs before reperfusion because it contains fructose and maintains slight acidity. Because Futhan is a strong protease inhibitor that has inhibitory activity on phospholipase A2 activation, it inhibits sinusoidal narrowing by blocking the metabolic pathway of arachidonic acid, maintaining the cytoskeleton, and inhibiting increases in the permeability of cell membranes. However, the details of the mechanisms remain unclear. Endothelin is a strong vasoconstrictor, increasing as blood flows to the portal veins; indeed the level of endothelin, which is derived from ischemic portal veins and sinusoids, may correlate with the degree of liver dysfunction. Therefore, it is reasonable to wash out the UW solution using a rinse containing the endothelin antagonists. In addition, it has been reported that neutrophil invasion is attenuated by the presence of endothelin antagonists during warm ischemia-reperfusion,[29] improving the survival rate after transplantation of livers from cardiac arrest donors.[30] There are also many reports on the effects of thromboxane A_2 (TXA_2) synthase inhibitors, since TXA_2 displays strong platelet aggregating and vasoconstrictive actions. Particularly, its vasoconstrictive action is thought to act directly on vascular smooth muscle. It is considered that TXA_2 is a factor that promotes the destruction of the vessel walls, because it increases local intravascular pressure. Since vascular smooth muscle does not exist in the hepatic sinusoids, TXA_2 is not directly involved in sinusoidal constriction. However, one report[31] observed TXA_2 receptors in sinusoidal endothelial cells, and that administration of a TXA_2 synthase inhibitor significantly inhibited the increased intravascular pressure suggesting that TXA_2 is related to sinusoidal constriction. Other reports state that TXA_2 synthase inhibitors inhibit neutrophil invasion and production of vasoconstrictors such as endothelin following reperfusion,[32,33] and TXA_2 synthase inhibitors were effective in liver transplantation from cardiac arrest donors.[34]

LIVER GRAFTS FROM CARDIAC ARREST DONORS

Recently, it has been reported that prostaglandin I_2[35] and prostaglandin E_1[36] stabilize cell membranes at the time of warm ischemia. These reports are based on observations in liver transplants from cardiac arrest donors. In fact, there are many studies involving liver transplantation from cardiac arrest donors, as well as warm ischemia-reperfusion injury in liver transplantation. The critical difference between liver transplantation from a cardiac arrest donor and from a heart-beating donor is whether the graft suffers warm ischemia during the agonal stage. The energy produced through the Krebs cycle is stored in cells as adenosin triphosphate (ATP), which maintains cell membrane functions including ion pumps. Anaerobic metabolism leads to hypofunction of the ion pump causing calcium to enter the cell, leading to phospholipase activation and production of arachidonic acid metabolites. Calcium inflow and potassium outflow produce swelling of the cells and their surrounding tissue. Cardiac arrest donors frequently experience hypotension, enteric congestion, and a decrease in pH during the agonal stage, events that promote endotoxin release and cytokine production.[37] Using rats, we investigated changes in cytokines and nuclear factors following reperfusion of liver grafts from cardiac arrest donors experiencing an agonal stage. The following increased levels were recognized: (1) cytokine (interleukin-1β) production; (2) the eicosanoid thromboxane and (3) particularly transcription factors, primarily NF-κB following reperfusion particularly after an agonal stage but not after old preservation alone. It has been reported that elimination of these Kupffer cells

abrogates changes attenuating the reperfusion injury.[38] When Kupffer cells were previously eliminated liver transplants from swine cardiac arrest donors showed remarkably attenuated, sinusoidal endothelial cell damage with narrowing, a pathologic event that was attenuated and long-term survival obtained[27] by a Futhan rinse. On the other hand, when the Futhan rinse was used but Kupffer cells were not removed, sinusoidal endothelial cell damage was not attenuated, although sinusoidal narrowing was attenuated. As a result, long-term survival was not obtained in this experiment. Therefore, it is thought that it is important to prevent two points in liver transplantation from cardiac arrest donors: sinusoidal endothelial cell damage and sinusoidal narrowing. Further studies are expected to clarify the events and mechanisms of these conditions to develop clinically applicable methods to prevent injury.

REFERENCES

1. Tsukamoto S, Ohkohchi N, Endoh T, et al: Transplant Proc 28:197, 1996
2. Gao WS, Takei Y, Marzi I, et al: Transplantation 52:417, 1991
3. Mckeown CMB, Edwards V, Phillips MJ, et al: Transplantation 46:178, 1988
4. Ohkochi N, Sakurada M, Koyamada N, et al: Tohoku J Exp Med 174:317, 1994
5. Sumimoto R, Lindell SL, Southard LH, et al: Transplantation 54:610, 1992
6. Tian Yh, Fukuda C, Schilling MK: Hepatology 28:1327, 1998
7. Gao W, Bentley RC, Madden JF, et al: Hepatology 27:1652, 1998
8. Sindram D, Kohli V, Madden JF, et al: Transplantation 68:136, 1999
9. Moriga T, Arii S, Takeda Y, et al: Transplantation 69:141, 2000
10. Caldwell-Kenkel JC, Currin RT, Tanaka Y, et al: Hepatology 10:292, 1989
11. Caldwell-Kenkel JC, Currin RT, Tanaka Y, et al: Hepatology 13:83, 1991
12. Arii S, Monden K, Adachi Y, et al: Transplantation 58:1072, 1994
13. Shibuya H, Ohkohchi N, Seya K, et al: Hepatology 25:356, 1997
14. Shibuya H, Ohkohchi N, Tsukamoto S: Hepatology 26:113, 1997
15. Schemmer P, Bradford BU, Rose ML, et al: Am J Physiol 276:G924, 1999
16. Ikejima K, Qu W, Stachlewitz RF, et al: Am J Physiol 272:G1581, 1997
17. Schemmer P, Schoonhoven R, Swenberg JA, et al: Transplantation 65:1015, 1998
18. Soejima Y, Yanaga K, Nishizaki T, et al: J Surg Res 86:150, 1999
19. Chen HM, Chen JC, Shyr MH, et al: Shock 12:462, 1999
20. Lentsch AB, Yoshidome H, Warner RL, et al: Gastroenterology 117:953, 1999
21. Harada N, Okajima K, Murakami K, et al: J Pharmacol Exp Ther 294:1034, 2000
22. Arai M, Mochida S, Ohno A, et al: Transplantation 62:1398, 1996
23. Yamanobe F, Mochida S, Ohno A, et al: Thrombo Res 85:493, 1997
24. Yoshimura N, Kobayashi Y, Nakamura K, et al: Transplantation 67:45, 1999
25. Mochida S, Arai M, Ohno A, et al: Hepatology 29:1532, 1999
26. Kaneko H, Joubara N, Yoshino M, et al: Eur Surg Res 32:87, 2000
27. Tsukamoto S, Ohkohchi N, Fukumori T, et al: Transplantation 67:1396, 1999
28. Tanaka W, Yamanaka N, Onishi M, et al: J Gastroenterol 35:120, 2000
29. Mitsuoka H, Suzuki S, Sakaguchi T, et al: Transplantation 67:514, 1999
30. Fukunaga K, Takada Y, Taniguchi H, et al: Transplantation 67:328, 1999
31. Ishiguro S, Arii S, Monden K, et al: Transplantation 59:957, 1995
32. Yokoyama I, Negita M, Kobayashi T, et al: J Surg Res 60:232, 1996
33. Yokoyama I, Kobayashi T, Negita M, et al: Transplant Int 9:76, 1996
34. Soejima Y, Yanaga K, Nisizaki T, et al: Surgery 123:67, 1998
35. Wang M, Sakon M, Miyoshi H, et al: J Surg Res 73:101, 1997
36. Kishimoto S, Sakon M, Umeshita K, et al: Transplantation 69:2314, 2000
37. Zhang Y, Ohkohchi N, Oikawa K, et al: Transplant Proc 32:3245, 2000
38. Oikawa K, Ohkohchi N, Sato M, et al: Transplant Int 13:s573, 2000