厚生労働科学研究費補助金（エイズ研究事業）
（分担）研究報告書

安全な生殖補助医療を行うためのHIVウィルス分離法の確立

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A. 研究目的

HIV 感染者の延命が可能になるとともに結婚して子供を望む夫婦が増えている。しかし、HIV 感染男性において精子の異常が高率に認められる。精子異常の原因は不明であるが、抗 HIV 剤や免疫低下、内分泌異常など多くの要因が考えられる。精子異常の原因を検討し、改善されれば生殖補助医療において良好な精子の選別が可能になる。精子研究により安全に子供を持てる方法を開発することを目的とする。

B. 研究方法

挙児希望で受診した HIV 感染男性に研究の目的と方法を文書で説明し、文書での同意を得たうえで実施する。荻窪病院で精液を採取し、精子数、運動率、正常形態率を測定する。血液検査で CD4 数、HIV RNA 量を測定する。精液を生理食塩水で洗浄し、2000 rpm で 10 分遠心して精子を分離し、RNA later を添加して保存する。

C. 研究結果

現在までに研究班に参加した 162 名の HIV 感染男性の精液所見を検討した。
全体の中央値（最低値～最高値）は、年齢 35（20～58）歳、精子数（濃度）5800 万（0～40700 万）/ml，運動率 40%(0~80%)，正常形態率 29%(0~81%)，CD4 数 410(41~1481)/ μl，HIV RNA 60(0~300000)copies/ml であった。
精子数が 2000 万以下の精子減少症を 31 名/162 名（19%）で認めた。
精子運動率が 50%以下の異常は 112 名/155 名（72%）で高率に認められた。
精子の正常形態率が 15%以下の異常は 48 名/145 名（33%）で認められた。

＃精子数減少群の検討

精子数が 2000 万以下の群（31 名）は、年齢 35.5（20～51）歳、精子数 600 万（0～2000 万）/ml，運動率 15%(0~80%)，正常形態率 13.5%(0~42%)，CD4 数 416（65~1030）/μl，HIV RNA 70(0~100000)copies/ml であった。
このうち、無精子症が 7 名認められ、7 名中 6 名が抗 HIV 治療を受けていた。中央値（最低値～最高値）は、年齢 37（20～51）歳、CD4 数 280（65~577）/ μl，HIV RNA 90(0~100000)copies/ml であった。

＃CD4 数の影響の検討

CD4 数が 400 未満/μl の群と 400 以上/μl の群に分けて比較検討した。
CD4 数 400 未満群は 73 名で、400 以上群が 85 名であった。
CD4 数 400 未満群の中央値（最低値～最高値）は、年齢 36（26～58）歳、精子数 7800 万（0～40700 万）/ml，運動率 45%(0~71%)，正常形態率 26.5%(0~74%)，CD4 数 300（41~399）/μl，HIV RNA <40(0~210000)copies/ml であった。
CD4 数 400 以上群の中央値（最低値～最高値）は、年齢 34（23～49）歳、精子数 5500 万（100～25700 万）/ml，運動率 40%(0~80%)，正常形態率 33%(2~81%)，CD4 数 525（400~1481）/μl，HIV RNA 90(0~300000)copies/ml であった。

#HIV RNA 量の影響の検討

HIV RNA 量が 1000 未満/ml の群と 1000 以上/ml の群に分けて比較検討した。
HIV RNA 量が 1000 未満群は 104 名で、1000 以上群が 54 名であった。
HIV RNA 量が 1000 未満群の中央値（最低値～最高値）は、年齢 36（25～58）歳、精子数 6400 万（0～40700 万）/ml，運動率 40%(0~75%)，正常形態率 25%(0~81%)，CD4 数 400（41~1481）/ μl，HIV RNA <40(0~720)copies/ml であった。
HIV RNA 量が 1000 以上群の中央値（最低値～最高値）は、年齢 35（23～47）歳、精子数 6500 万(100～23400 万)/ml，運動率 46.5%(3~80%)，

正常形態率 34.5％(3~68%), CD4 数 439 (60~1100) / μl, HIV RNA 10000(1000~300000)copies/ml であった。

＃抗HIV剤の影響に関する検討
抗 HIV 剤使用の有無による精子への影響を検討した。
抗HIV剤を使用している群は114名で、無治療群が51名であった。
抗 HIV 剤使用群の中央値（最低値～最高値）は、年齢35（20～58）歳、精子数5400万（0～40700万）/ml, 運動率40%(0~75%), 正常形態率31.5%(0~81%), CD4数398（41~1481）/μl, HIV RNA <40(0~210000)copies/ml であった。
無治療群の中央値（最低値～最高値）は、年齢35（23～47）歳、精子数7000万（0～23400万)/ml, 運動率45%(3~80%), 正常形態率31%(3~68%), CD4数492(66~1216)/ μl, HIV RNA 6400(70~300000)copies/ml であった。

D. 考察
HIV 感染者では精子数が少なく、運動率が低く、正常形態率も低かった。精子濃度減少と年齢、HIV RNA 量との相関は認められなかった。CD4数が高いと精子数が少なく、CD4 数が低いと正常形態率が低くなる傾向が認められた。HIV RNA 量が低いと運動率が低く、正常形態率が低くなる傾向が認められた。抗HIV剤使用群では無治療群に比較して精子の数が少なく、運動率が低かった。以上から、抗HIV剤の使用によりHIV RNA 量が低くなり、CD4 数が高くなると精子への影響が強まる可能性が考えられた。HIV 感染者の精子の異常は運動率低下が著明であった。投薬を受けている一部の感染者において精子を電子顕微鏡で検討したところ、精子のミトコンドリアが高度に障害されていた。抗HIV剤により体細胞のミトコンドリアが障害され糖脂質代謝異常などが生じることは知られているが、精子も抗HIV剤により障害されていることが示唆された。精子のミトコンドリアが障害されると精子の運動率が低下すると考えられる。精子運動率が低いと男性不妊となる場合もある。今後、抗HIV剤使用に伴うミトコンドリア障害について精査する必要がある。

E. 結論
1) 研究成果の学術的・国際的・社会的意義について
HIV感染者は世界中で増加しており、先進国においても新規感染者が多い。一方治療の進歩により感染者は延命可能となり、QOLの改善を求めており、結婚して子どもを望む夫婦も増えている。HIV感染者の精子の検討は世界中で不十分で、抗 HIV 剤などの影響も解明されていない。
2) 今後の展望について
今後、各薬剤毎の検討や詳細なミトコンドリア研究を加えることにより、免疫不全や抗HIV剤の精子への影響を検討する。それによって生殖医療の検討に有益となる。

G. 研究発表
1.論文発表
Kinai E, Hanabusa H.: Progressive renal tubular dysfunction associated with long-term use of tenofovir DF. AIDS Res Hum Retroviruses. 2009 Apr;25(4):387-94.
花房秀次: HIV感染者の生殖補助医療、感染・炎症・免疫(0387-1010)39巻1号 Page52-55, 2009
木内英, 花房秀次: 【Emergency 実戦ガイド】疾患と対応 血液 血友病患者における出血 内科, 103巻6号 Page1620-1626, 2009
木内英, 花房秀次: 【小児感染症のすべて】感染症の種類別に小児感染症を考える 小児の HIV 感染症. 化学療法の領域, 25 巻増刊 Page995-1005, 2009
花房秀次: HIV/AIDS の現状と HIV 陽性夫婦の生殖補助医療 HIV 完全除去精子を用いた体外受精、夫婦共に HIV 陽性の場合における superinfection 防止、母子感染防止対策など. 臨牀と研究、86巻9号 Page1219、2009
学会発表
木内英, 小島賢一, 和田育子, 石倉美緒, 花房秀次: HIV感染者の腎機能評価とシスタチンC の有用性に関する検討日本エイズ学会(2009.11)
花房秀次, 木内英, 和田育子, 石倉未緒, 小島賢一: 多剤耐性 HIV 感染者における Raltegravir 長期使用における有効性と安全性の検討, 日本エイズ学会 (2009.11)
荒木辰也, 金栗沙耶香, 友清和彦, 石原範之, 中垣智弘, 木内英, 花房秀次: 第VIII因子製剤固有のインヒビター価測定法の検討, 日本血栓止血学会 (2009.04)
福武勝幸, 白幡聡, 高松純樹, 竹谷英之, 花房秀次, 日笠聡, 藤井輝久, 松下正, 松本剛史, 三間屋純一, 大西赤人, 血友病在宅治療

支援プログラム委員会：患者・家族を対象とした血友病在宅治療状況調査の成績，日本血栓止血学会（2009.04）
日笠聡，新谷憲治，花房秀次，毛利博，天野景裕，岡敏明，酒井道生，白幡聡，高田昇，高松純樹，瀧正志，竹谷英之，田中一郎，福武勝幸，藤井輝久，松下正，三間屋純一，三室淳，吉岡章，嶋緑倫，日本血栓止血学会学術標準化委員会血友病部会：後天性血友病 A の免疫抑制療法ガイドライン案，日本血栓止血学会（2009.04）
松下正，酒井道生，藤井輝久，家子正裕，新谷憲治，山崎雅英，天野景裕，瀧正志，岡敏明，白幡聡，高田昇，高松純樹，竹谷英之，花房秀次，日笠聡，福武勝幸，田中一郎，三間屋純一，三室淳，吉岡章，嶋緑倫，日本血栓止血学会学術標準化委員会血友病部会：後天性血友病 A の止血療法ガイドライン(案)，日本血栓止血学会（2009.04）
天野景裕，瀧正志，家子正裕，山崎雅英，岡敏明，酒井道生，白幡聡，高田昇，高松純樹，竹谷英之，田中一郎，花房秀次，日笠聡，福武勝幸，藤井輝久，松下正，三間屋純一，三室淳，吉岡章，嶋緑倫，日本血栓止血学会学術標準化委員会血友病部会：後天性血友病 A の診断ガイドライン案，日本血栓止血学会（2009.04）

H.　知的所有権の出願・取得状況
現在のところ予定なし。

厚生労働科学研究費補助金（エイズ対策研究事業）
分担研究報告書

安全な生殖補助医療を行うための精液よりのHIVウイルス分離法の確立

分担研究
ヒト精子凍結保存の最適化に関する研究

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A. 研究目的

本研究は、HIV陽性患者精液を分画してウィルス除去した運動精子を調製し、これを用いた生殖補助医療(ART)により挙児を図ることを目的としている。これを達成するための技術要素として1. 精液からの運動精子とHIVの分離法の開発、2. 分画した精子懸濁液中のHIV陰性確認（高感度HIV検出）、3. 項目2における検査時間の確保のため精子を凍結保存し、一部と使用して検査を行うことが不可欠である。項目1において妊孕性の高い成熟した運動精子分画における回収率は必ずしも高くなく、また項目2における検査に半量を使用するため、凍結保存に供する精子の量は少量となってしまう。さらに精子保存における凍結、融解過程における精子蘇生率（融解後における運動精子回収率）の低さがART臨床応用へのボトルネックとなっている。

ヒト精子凍結保存は約50年の歴史を有し、広く臨床応用されている。グリセリンを凍結保護剤とする保存液が汎用され、精子凍結保存に関する手法はすでに完成されたものと考えられてきた。一方、精子凍結保存に際して、融解後蘇生率に個体差が存在することは広く知られ、それは凍結手技が最適化されていないからなのか、運動精子には耐凍性の高いものと低いものが混在し、それらの比率に個体差が存在するためなのか、不明であった。

精液中のHIVは浮遊ウィルスとリンパ球内に大別される。前者を除去する過程において精漿の完全除去が求められる。洗浄後、精子は精漿から人工培養液に置換されるが、この過程における体液タンパク質除去に伴うコロイド浸透圧の低下が精子蘇生率の低下の主因であることが明らかとなった。

本研究は凍結保存精子の妊孕性向上を目的とし、凍結保護剤、凍結条件、融解後の保護剤除去に関して最適化を行った。

B. 研究方法

精子の分画には、1.クッション法による精子濃縮、2.Optidenz 沈降平衡、3.Percoll 沈降速度差遠心分離法を用いた。凍結保護剤として、メタノール、グリセリン、エチレングリコール、DMSOを

検討した。凍結には、すでに報告した可変型 2 重空容器を用い、液体チッソに直接投入する簡易凍結法を用いた。保存後、液体チッソから取り出した容器を開封し、内容器を取り出して 37℃の温湯中で融解した。最終的に融解剤を添加した培養液で希釈し、精子内の凍結保護剤を除去した。

（倫理面への配慮）

東京歯科大学市川総合病院リプロダクションセンターを受診し、精液検査を施行した検体のうち、研究使用へのインフォームドコンセントが得られた精液を使用した。

C. 研究結果

1. 本年度は等倍濃度の保護剤を予め添加したコロイドシリカゲル担体（Percoll 等）を用いて洗浄を行う方法を検討した。図 1 は各過程における分離の実際を示している。これまでは予め洗浄濃縮した精子懸濁液に 2 倍濃度の凍結保護剤を等量添加、混合した後、平衡化（保護剤の細胞内浸透）を行った。本法は遠心分離（約 30 分間）と平衡化が同時進行し、平衡化時間を長く取ることができる。遠心後の沈澱はすでに保護剤が浸透した状態であり、直ちに容器に収納して凍結を行うことができる。さらに保護剤添加による希釈を回避できる利点を有する。
2. 凍結保護剤処方を検討した結果、Hanks 等張化 80% Percoll-Plus 液に 50mM トレハロース、150mM DMSO、150mM エチレングリコールを添加した時、最も高い蘇生率が得られた。従来、汎用されてきたグリセリンは至適な保護物質ではなかった。本処方では将来的に使用禁止となる卵黄等の異種因子を排除しており、chemical defined にできた。遠心分離は、400xg、30 分間行った。
3. 内容器に回収した沈澱精子（約 100 μl）を入れ、外容器に収納して直ちに液体チッソに投入した。本容器は断熱性が高く、液体チッソ蒸気内に容器を懸垂して緩速凍結を行うことなく、精子生存性が確保できた。今後、胚、精子を問わず、保存中液体チッソに直接触れることは許可にならない方向で規制を受ける。本研究で開発した可変型 2 重空チューブは、密閉容器内に密閉容器を入れることで検体が液体チッソに全く触れる可能性は極めて低くなった。
4. 一般に精子凍結保存に関する研究は、融解直後の運動率（蘇生率）を指標として検討することが多い。項目 1-3 にまとめたように、精子を凍結、融解するまでの過程においては満足すべき結果が得られた。凍結保存精子を ART に使用するには、精子から凍結保護剤を除去して最終的に培養液に置換する必要がある。これまでの検討において、平衡塩類溶液に少量のアルブミンを添加した既存の培養液では、保護剤の除去に伴い運動能低下とともに尾部細胞膜の浮腫が生じ、これが妊孕性低下の主因であると考察された。その防止にはコロイド浸透圧の付加が不可欠であると考え、培養液へのヒドロ

キシエチルデンプン、ポリエチレングリコール、細胞膜非透過糖類、タンパク質（アルブミン、血清、卵胞液）を添加し、融解精子をこれらで希釈した場合の尾部浮腫発現を観察した。検討した全てのコロイド物質において、浮腫抑制効果を確認した。糖由来の高分子よりもさらにタンパク質の有効性が高いことを確認した。血清もしくは卵胞液自体を培養液として使用することにより、上述した運動率の低下、尾部浮腫の出現を阻止できることが明らかとなった。

5. 上述した基礎的検討を踏まえて精子凍結保存を試みた。射精精液から調製した洗浄精子（n=5）の凍結融解前後の運動率は、各々88±6.8%、62±7.0%であった。ハンクス液を用いて凍結保護剤除去を行うと、運動率は 4.6±3.7%へと大幅に低下し、不働化した精子に多様な尾部異常、とくに先端に特有な浮腫を認めた。一方、血清を用いた場合は 49±7.4%の精子が、さらに 0.2M トレハロースを添加した 67%血清を用いて希釈した場合には尾部浮腫をほとんど認めず、54±6.2%の精子が前進運動性を保持していた。コロイド浸透圧の意義が確認されるとともに複数の物質を組み合わせることによる相乗効果を認めた。

D. 考察

1. 凍結保護剤として汎用されてきたグリセリンは、親水性が高く細胞膜を通らない（グリセリンは脳浮腫などにおいて除圧に使用され、細胞透過性は低い）。ヒト精子にはグリセリン透過性アクアポリンチャネルが発現しており、細胞膜の脂質2重層を直接かつ高速に透過するメタノール、エチレングリコール、DMSO 等の低分子両親媒性物質と比較して速度は遅いが、少しずつ細胞内に蓄積して保護効果を発揮する。
2. 卵黄の作用機序はリン脂質の精子細胞膜被覆と考えられてきたが、保存液を調製する過程で遠心分離すると脂質成分は沈澱し、上清、すなわち保護剤の成分として残留するのは水溶性のたんぱく成分が主となる。精子凍結融解過程においては、コロイド浸透圧が重要な保護因子であり、卵黄タンパク質はコロイドとして作用している可能性が示唆された。
3. 結果の項目2で述べたように、細胞浸透性が高く、作用機序の異なるエチレングリコール、DMSO の共沸混合物を組み合わせた時、融解直後の残存運動率は最も高かった。メタノールも凍結保護効果は優れていたが、毒性の観点から使用しないこととした。
4. ART に使用するためには保護剤を除去して培養液に置換しなくてはならない。一般的な胚培養液は塩溶液に少量のアルブミンを添加したものが多い。これらの培養液は、コロイド浸透圧が低く、希釈により外環境の保護剤濃度が低下すると、この過程で細胞浮腫、細胞膜の障害が誘起する。すなわち、凍結保存における凍結、融解、最終的な保護剤除去の段階の内、最後の保護剤除去が最も細胞障害性が高いこと

が明らかとなった。胚凍結保存における融解時は、高張ショ糖溶液のドロップ内を順次移していく手法が採られるが、精子においても同様なプロセスが重要である。現在、血清等の体液に高分子糖類を添加した歳に相乗効果を認めたので、どの組み合わせ、濃度が最適化か検討を行っている。

E.　結論

本年度はヒト精子凍結保存に関して詳細な検討を行い、凍結、融解、最終的な保護剤除去の各過程で最適化を行うことが凍結保存精子の ART 臨床応用に不可欠であることが明らかとなった。

F.　健康危険情報

該当なし。

G.　研究発表

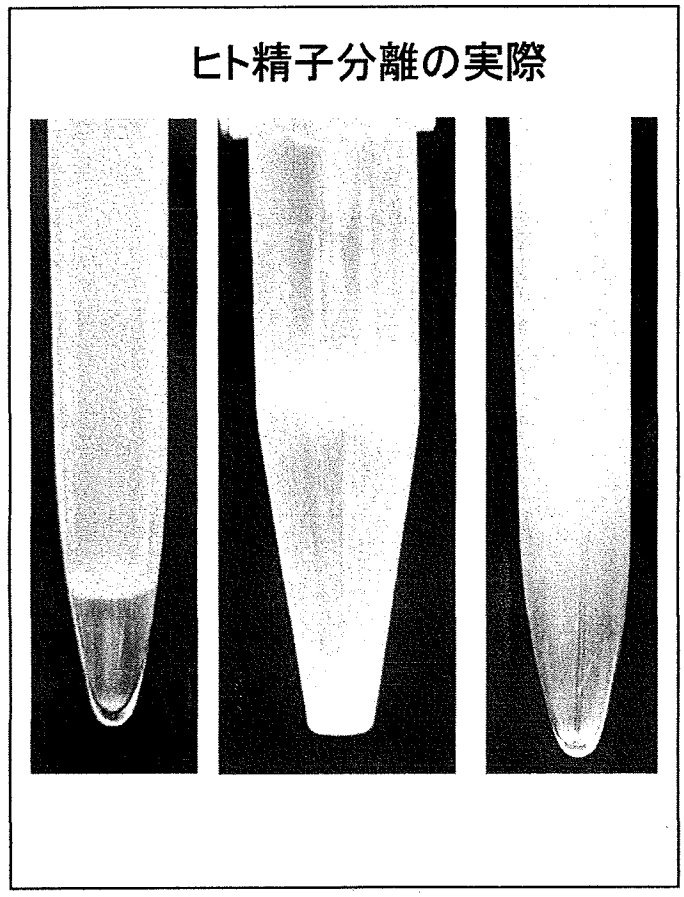
学会発表

1.　原唯純、兼子智、石川博通、高松　潔、佐藤博久：　コロイド浸透圧を考慮したヒト精子凍結、融解システムの構築、第140 回、日本生殖医学会関東地方部会、2009

付図

ヒト精子分離の実際

左からクッション法による精子濃縮、Optidenz 沈降平衡、Percoll-Plus 沈降速度差遠心分離を示している



厚生労働科学研究費補助金（エイズ対策事業）
分担研究報告書

中空糸膜ウイルス除去カラムによる、より効率的な精液中HIV除去方法の開発

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研究要旨
中空糸血漿分離膜を用いた精液中からのエイズウイルス分離研究を広く告知するためにホームページからの情報発信を行うとともに、研究に用いる中空糸血漿分離膜の提供を行った。

A. 研究目的

精液からの HIV 除去を簡便に実施でき、受精の確率を高めることが予想されるHIV除去法の確立

B. 研究方法

旭化成クラレメディカルが提供する中空糸血漿分離膜に精液懸濁液を通過させることによりHIVのみが除去される。この操作を繰り返すことにより受精可能な精液が精製される。

C. 研究結果

本年度は研究の内容をホームページにまとめる作業を行うとともに中空糸血漿分離膜10本の提供を行った。

D. 考察

HIV が混入している可能性のある、精液中白血球の除去法として、本処置の前に、白血球除去能力のある不織布に浸す対応が考えられた。

E. 結論

ホームページでの情報公開を行い、中空糸血漿分離膜の提供を行った。

F. 健康危険情報

なし

G. 研究発表

1．論文発表
なし
2．学会発表
なし

H. 知的財産権の出願・登録状況
（予定を含む）

1．特許取得
なし
2．実用新案登録
なし
3．その他
なし

発表者氏名	論文タイトル名	発表誌名	巻号	頁	出版年
Kashima. K , Takakuwa, K., Suzuki, M., Makino, M., Kaneko S,, Kato, S., Hanabusa, H., Tanaka, K.	Studies on assisted reproduction techniques (ART) for HIV-1 discordant couples using washed sperm and the nested PCR method -Comparison of the pregnancy rate in HIV-1 discordant couples and control	Jpn. J. Infect. Dis.	62	173-176	2009
Serikawa, T., Takahashi, Y., Ichikawa, K., Uemura, R., Kikuchi, A., Takakuwa, K., Sakakibara, S., Matsunaga, M., Tanaka, K.	A case of neonatal alloimmune thrombocytopenia from human platelet antigen 5b incompatibility	Reprod. Immunol. Biol.	24	18-20	2009
Serikawa, T., Ichikawa, K., Kikuchi, A., Takakuwa, K., Tanaka,K.,	A case Study of a Pregnant Patient with a Congenital Heart Block Accompanied by Left Isomerism and Uncontrolled Type 2 Diabetes Who Was Treated Successfully with Ritodrine	Gynecologic and Obstetric Investigation	69	193-196	2010
Sasahara, J., Kikuchi, A., Takakuwa, K., Sugita, N., Abiko, Y., Yoshie, H., Tanaka, K.	Antibody response to Porphyromonas gingivalis outer membrane protein in the first trimester	Aust. N. Z. J. Obstet. Gynaecol.	49	137-141	2009
Yamaguchi, M., Kikuchi, A., Ohkusu, K., Akashi, M., Sasahara, J.,Takakuwa, K., Tanaka, K.	Abscess formation due to Mycoplasma hominis infection after cesarean section	J. Obstet. Gynaecol. Res.	35	593-596	2009
Nonaka, T., Kikuchi, A., Kido, N., Takahashi, Y., Yamada, K., Usuda, T., Takakuwa, K., Tanaka, K.	Prenatal diagnosis of unilateral pulmonary agenesis in a pregnant woman undergoing chronic hemodialysis due to chronic renal failure	Prenat. Diagn.	29	1078-1080	2009

Yamada, K., Takakuwa, K., Tekeyama, S., Minagawa, S., Morikawa, H., Matsunaga, M., Tomita, M., Tanaka, K.	A case of fulminant yype 1 diabetes mellitus that acutely emerged during pregnancy	J. Obstet. Gynaecol. Res.		in press	2010
Wada, Y., Sakamaki, Y., Kobayshi, D., Ajiro, J., Moro, H., Murakami, S., Ooki, I., Kikuchi, A., Takakuwa, K., Tanaka, K., Sato, T., Nakano, M., Narita, I.	HELLP syndrome, multiple liver infarctions, and intrauterine fetal death in a patient with systemic lupus erythematosus and antiphospholipid syndrome	Intern. Med.	48	1555-1558	2009
高桑好一	新型(豚)インフルエンザへの対応、5. インフルエンザ流行時における妊婦への対応	化学療法の領域	25	2082-2086	2009
高桑好一	周産期医療とInflammatory response -歯周病-	周産期医学	39	745-749	2009
Quan, J., Yahata, T., Tamura, N., Nagata, H., Tanaka, K.	Relationship between single nueleotide polymorphisms in CYP1A1 and CYP1B1 genes and the bone mineral density and serum lipid profiles in postmenopausal Japanese women taking hormone therapy	Menopause	16	171-176	2009
Kinai E, Hanabusa H.	Progressive renal tubular dysfunction associated with long-term use of tenofovir DF	AIDS Res Hum Retroviruses	25	387-394	2009
花房秀次	HIV感染者の生殖補助医療	感染・炎症・免疫	39	52-55	2009
木内英, 花房秀次	疾患と対応 血液 血友病患者における出血	内科	103	1620-1626	2009
木内英, 花房秀次	感染症の種類別に小児感染症を考える　　小児のHIV 感染症	化学療法の領域	25	995-1005	2009
加藤真吾、今井光信	HIV検査と検査相談体制	最新医学・別冊 新しい診断と治療のABC 65		180-87	2010

Kondo, M., Sudo, K., Tanaka, R., Sano, T., Sagara, H., Iwamuro, S., Takebe Y., Imai, M., and Kato, S	Quantification of HIV-1 group M proviral DNA using TaqMan MGB real-time PCR	J. Virol. Methods	157	141-146	2009
Suzuki, T., Yamamoto, N., Nonaka, M., Hashimoto, Y., Matsuda, G., Takashima, S., Matsuyama, M., Igarashi, T., Miura, T., Tanaka, R., Kato, S., and Aida, Y.	Inhibition of human immunodeficiency virus type 1 (HIV-1) nuclear import via Vpr-Importin α interaction as a novel HIV-1 therapy	Biochem. Biophys. Res. Commun.	380	838-843	2009
Shima-Sano, T., Yamada, R., Sekita, K., Hankins, R. W., Horr, H., Seto, H., Sudo, K., Kondo, M., Kawahara, K., Tsukahara, Y., Inaba, N., Kato, S., Imai, M.	A human immunodeficiency virus screening algorithm to address the high rate of false-positive results in pregnant women in Japan.	PLoS One	5	e9382	2009
加藤真吾	HIV検査法の現状と課題	病原微生物検出情報	30	236-237	2009
今井光信,加藤真吾	HIV検査—最近のスクリーニング検査と遺伝子検査の進歩—	日本臨床	68	433-438	2010

Original Article

Studies of Assisted Reproduction Techniques (ART) for HIV-1-Discordant Couples Using Washed Sperm and the Nested PCR Method: a Comparison of the Pregnancy Rates in HIV-1-Discordant Couples and Control Couples

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SUMMARY: In this study, the efficacy and safety of assisted reproduction techniques with the sperm-washing method and nested PCR assay were evaluated in HIV-1-discordant couples, as many HIV-1-positive people of reproductive age are getting married and wish to have children safely. Twenty-seven HIV-1-discordant couples (husband, positive; wife, negative) were enrolled in this study. The spermatozoa were separated from semen samples by density gradient centrifugation and the swim-up method. HIV-1 RNA and proviral DNA were checked using nested PCR with a detection limit of one copy before fertilization and before embryo transfer. Clinical outcomes were compared with those of matched control couples. Thirty-eight cycles of in vitro fertilization or intracytoplasmic sperm injection were performed in HIV-1-discordant couples, where the pregnancy rates per embryo transfer and per couple were 60.6 and 63.0%, respectively. These rates were significantly higher than those in control couples ($P < 0.05$). Furthermore, all of the females and babies remained HIV-1 negative throughout the study period. Our data strongly suggest that this technique will allow HIV-1-discordant couples to conceive more safely and effectively.

INTRODUCTION

Recently, people infected with human immunodeficiency virus-1 (HIV-1) have been living longer, since the application of highly active antiretroviral therapy (HAART) has greatly improved survival. Many HIV-1-positive people of reproductive age are getting married and wish to have children safely. It would be possible for an HIV-1-infected male to father children without the risk of HIV-1 transmission if HIV-1-free spermatozoa could be obtained from his semen. The clinical value of sperm washing as well as its risks was first reported in 1992 by Semprini et al. (1), and since then, it has been confirmed by many authors examining both methodological issues and clinical data. In this study, we applied the assisted reproduction technique (ART) for HIV-1-discordant couples in which the man was HIV-positive and the woman was negative, using the swim-up method and nested polymerase chain reaction (PCR) assay and tried to elucidate the efficacy and safety of the procedure.

MATERIALS AND METHODS

Patient couples: First, the patients consulted the Department of Hematology of Ogikubo Hospital, and the HIV-1 infection status of the husband was assessed. At this time, couples were also informed of the details of this treatment by one of the doctors in the study group. After the couples confirmed their desire to undergo the treatment, they were referred to Niigata University Hospital and then were again informed of the details of the treatment by another doctor in the study group with a counselor. The explanation included details of the procedure for ovulation induction, oocyte retrieval, and embryo transfer as well as the risks of these procedures, followed by an explanation of the protocol for confirming the elimination of HIV-1 from the husband's semen. The risk of secondary HIV-1 infection to both mother and baby, if the wife were to conceive, was also thoroughly explained. After the patients confirmed the final decision to participate in this study and gave written informed consent to treatment, the treatment was started. The approval of the ethical committee of Niigata University School of Medicine was obtained.

Semen pretreatments: Semen samples were obtained by masturbation and then tested for sperm concentrations, motility, and deformity. An improved swim-up method was used to collect HIV-1-free spermatozoa from the semen of HIV-1-positive males. Diluted semen was layered over a Percoll solution with a continuous density gradient of 30 - 98% and then centrifuged. We collected the sperm fraction from the end of the tube, and the spermatozoa were collected using the swim-up method as previously described (2). The sperm suspension was divided into two portions, and one half was provided for HIV-1 assessment while the other was cryopreserved in a liquid nitrogen container.

Detection of HIV-1 RNA and proviral DNA: The HIV-1 RNA and proviral DNA were measured by the nested PCR

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method as previously described (2), with a detection limit of one copy. The oocytes obtained from the HIV-1-negative wives were fertilized after confirming that HIV-1 could not be detected in the washed semen samples by the nested PCR method. Furthermore, the fertilized eggs were cultured for 2 or 3 days and were transferred after a negative result was obtained by the nested PCR procedure in the culture medium of the fertilized eggs.

In vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI): The standard long protocol was adopted for most ovulation stimulation cycles. The short protocol was used for patients who were poor responders. If HIV-1 testing for virion RNA and proviral DNA was negative, the other portion of the sperm sample was thawed for use in conventional IVF or ICSI. IVF or ICSI was offered according to the semen profile of each male. The embryo transfer was conducted only when HIV-1 RNA and proviral DNA were negative by the nested PCR assay.

Seroconversion tests: All female partners who underwent ART, even those who did not conceive successfully, were tested for HIV antibodies, HIV-1 RNA, and proviral DNA in the blood 1, 2, and 3 months after the embryo transfer. Furthermore, babies born to the mothers were tested for HIV-1 RNA and proviral DNA at birth or later.

Control couples: Control couples matched to the woman's age who underwent conventional IVF or ICSI in Niigata University Hospital between January 2001 and July 2007 were randomly selected to assess clinical efficacy. During this period, 417 patients aged between 24 and 48 years underwent 814 cycles of IVF or ICSI by the long or short protocol. Within these control couples, 465 cycles of IVF from 261 patients (403 embryo transfers) and 209 cycles of ICSI (182 embryo transfers) from 118 patients were used to compare clinical results to those of HIV-1-discordant couples undergoing embryo transfer.

Statistical analyses: The Student's t test and chi-square test were used to test differences between HIV-1-discordant couples and control couples. Significance was defined as $P < 0.05$.

RESULTS

Twenty-seven discordant couples in which the man was HIV-1 positive and the woman was negative were enrolled in this study at Niigata University Hospital between January 2001 and July 2007. The age of the women ranged from 21 to 41, with a mean of 32.3 years. Of the 27 males, the plasma HIV-1 viral load was <50 copies/ml in 15 patients, and the median plasma HIV-1 viral load of the other 12 was 967 copies/ml (range, 100 - 100,000). In addition, the median CD4 cell count was 377 cells/ml (range, 96 - 700) in 27 patients.

Twenty-seven women underwent ovulation induction 38 times. Of these 27 patients, 5 underwent ovulation induction twice, and another 2 underwent ovulation induction 3 and 5 times, respectively. The remaining 20 patients each underwent induction once. Two cycles were cancelled due to poor response. HIV-1 RNA and proviral DNA were not detected by the nested-PCR assay in any of the 36 of spermatozoa samples collected from 27 patients. HIV-1-negative sperm were used for IVF in 12 couples and for ICSI in 18 couples. To date, fertilized eggs were obtained in 26 women and embryo transfer was performed in all 26 women after confirming that HIV-1 RNA and proviral DNA could not be detected in the culture medium of the fertilized eggs. Three cycles were canceled due to the lack of fertilization.

The clinical pregnancy rate per embryo transfer was 60.6% (Table 1). Of the 27 HIV-1-discordant couples, 17 patients (63.0%) conceived and 22 babies were born. Three cases resulted in early abortion. The multiple pregnancy rate was 25.0%, with 4 sets of twins and 1 set of triplets. HIV-1 RNA and proviral DNA were negative in all of the females and infants throughout the study period. The median observation period of born babies was 58 months (range, 10 - 86). The clinical pregnancy rates per embryo transfer and per couple in the control couples were 30.8% (180 of 585) and 42.5% (161 of 379), respectively. Therefore, the clinical pregnancy rate per embryo transfer as well as that per couple in HIV-discordant couples was significantly higher compared with that in control couples ($P < 0.001$ and $P < 0.05$ by chi-square test, respectively).

Thirteen cycles of IVF were performed in 12 couples, with the clinical pregnancy rates per cycle and per couple being 72.7 and 66.7%, respectively, with a multiple pregnancy rate of 37.5% (Table 2). There were 23 cycles in 18 couples treated by ICSI, with the clinical pregnancy rates per cycle and per couple being 54.5 and 55.6%, respectively, with a multiple pregnancy rate of 16.7% (Table 3). Although the clinical pregnancy rate was higher in the IVF couples, the difference was not significant.

In the control patients, the clinical pregnancy rates per

Table 1. Clinical outcomes of IVF/ICSI cycles in 27 couples

	Total	Range
Couples (n)	27	
Age (y)	32.3 ± 5.0	21 - 41
Cycles (n)	38	
Total gonadotropin dose (IU)	2,022.4 ± 777.4	1,050 - 4,200
Retrieved oocytes (n)	9.8 ± 6.2	1 - 22
Fertilization rate (%)	50.6 (179/354)	
Transferred embryos (n)	2.2 ± 0.6	1 - 3
Implantation rate (%)	34.8 (24/69)	
Clinical pregnancy rate per embryo transfer (%)	60.6 (20/33)	
Clinical pregnancy rate per couple (%)	63.0 (17/27)	
Delivered pregnancy rate (%)	85.0 (17/20)	
Multiple pregnancy rate (%)	25.0 (5/20)	
Maternal seroconversion (n)	0	
Delivered offspring seroconversion (n)	0	

IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection.

Table 2. Comparison of clinical outcomes between HIV-1-discordant couples and control couples undergoing conventional IVF

	HIV couple	Control couple	*P*-value
Couples (n)	12	261	
Age (y)	31.2 ± 5.0	34.2 ± 3.5	not significant
Cycles (n)	13	465	
Retrieved oocytes (n)	10.8 ± 7.6	6.8 ± 4.8	
Fertilization rate (%)	57.4 (74/129)	71.4 (2,269/3,178)	<0.01
Transferred embryos (n)	2.4 ± 0.5	2.2 ± 0.8	not significant
Implantation rate (%)	42.3 (11/26)	16.0 (140/877)	<0.01
Clinical pregnancy rate per embryo transfer (%)	72.7 (8/11)	30.3 (122/403)	<0.01
Clinical pregnancy rate per couple (%)	66.7 (8/12)	42.1 (110/261)	not significant
Delivered pregnancy rate (%)	100.0 (8/8)	74.6 (91/122)	not significant
Multiple pregnancy rate (%)	37.5 (3/8)	12.3 (15/122)	not significant

Table 3. Comparison of clinical outcomes between HIV-1-discordant couples and control couples undergoing ICSI

	HIV couple	Control couple	*P*-value
Couples (n)	18	118	
Age (y)	33.7 ± 4.5	35.6 ± 3.9	not significant
Cycles (n)	23	209	
Retrieved oocytes (n)	9.7 ± 5.6	7.9 ± 4.9	not significant
Fertilization rate (%)	47.3 (105/222)	49.8 (823/1,653)	not significant
Transferred embryos (n)	2.0 ± 0.7	2.0 ± 1.1	not significant
Implantation rate (%)	28.9 (13/45)	15.6 (64/411)	<0.05
Clinical pregnancy rate per embryo transfer (%)	54.5 (12/22)	31.8 (58/182)	<0.05
Clinical pregnancy rate per couple (%)	55.6 (10/18)	43.2 (51/118)	not significant
Delivered pregnancy rate (%)	75.0 (9/12)	81.2 (47/58)	not significant
Multiple pregnancy rate (%)	16.7 (2/12)	10.3 (6/58)	not significant

embryo transfer of IVF and ICSI were 30.3 and 31.8%, respectively (Tables 2 and 3). The implantation rate and the clinical pregnancy rate per embryo transfer were significantly higher in the HIV-1-discordant couples, especially for IVF treatment (The *P*-values appear in Tables 2 and 3; statistical analyses were performed using the chi-square test).

DISCUSSION

The heterosexual transmission rate of the HIV-1 virus is not very high, but a risk does exist. The rate of male-to-female transmission of HIV-1 in stable heterosexual relationships is estimated to be approximately 1 per 1,000 acts of unprotected intercourse (3). The transmission rates are perhaps greater with advanced stages of the disease, the presence of ulcerative genital infection, a history of previous sexually transmitted disease in the female partner, and the presence of postcoital bleeding. Araneta et al. have reported that the risk of transmission with intrauterine insemination (IUI) using non-washed semen from an infected man is 3.52% (4). For HIV-1-discordant couples with male infection, techniques such as sperm washing would further reduce the risk of transmission. The clinical value of sperm washing and the absence of seroconversions were first reported in 1992 (1). Since then, the method has been confirmed with regard to clinical issues by many authors (5-10). They have reported pregnancy rates per IUI cycle ranging from 15 to 31% (Table 4). However, their method may be suboptimal because it has not been proven to remove HIV-1 RNA completely, and they have not checked proviral DNA in infected cells in the semen. Zhang et al. have reported that HIV-1 may be present as proviral DNA in seminal cells in HIV-1-infected men who have achieved undetectable levels of viral RNA in plasma with HAART (11), and this HIV-1 could be transmitted sexually.

Table 4. Results of ART in HIV-1-discordant couples with infected-male partner

Reference	Couple	Cycle	Pregnancy (%)
IUI treatment			
Semprini et al. (1997)	350	1,000	200 (20.0)
Marina et al. (1998)	63	101	31 (30.7)
Weigel et al. (2001)	47	101	15 (14.9)
Bujan et al. (2004)	56	213	37 (17.4)
Nicopoullos et al. (2004)	105	133	25 (18.8)
Savasi et al. (2007)	581	2,400	456 (19.6)
IVF or ICSI treatment		(embryo transfer)	
Weigel et al. (2001)	19	31	15 (48.4)
Sauer et al. (2002)	34	55	25 (45.5)
Pena et al. (2003)	61	100	35 (35.0)
Ohl et al. (2003)	47	41	20 (48.8)
Chu et al. (2005)	92	146	54 (37.0)
Savasi et al. (2007)	160	278	65 (23.0)
Present study	27	33	22 (60.6)

ART, assisted reproduction technique; IUI, intrauterine insemination.

Although IUI therapy may be simpler and less expensive than IVF or ICSI therapy, IVF or ICSI involves a lower exposure of sperm cells compared to that in IUI, which requires millions of sperm to be placed in the uterine cavity. Another advantage of IVF or ICSI over IUI relates to the increase in pregnancy rates per treatment cycle. IVF or ICSI should decrease the number of attempts needed to establish a success-

ful pregnancy, thus further reducing potential viral exposure from repetitive treatment cycles. Since 1998, several groups have reported the results of IVF or ICSI in HIV-1-discordant couples with an HIV-1-infected male partner (3,7,10,12-16). The pregnancy rates reported in these studies were higher than those obtained using the IUI technique (Table 4). In the present study, the pregnancy rates per embryo transfer of IVF and ICSI were 72.7 and 54.5%, respectively. These data were significantly better than those of the control group for both IVF and ICSI treatment (Tables 2 and 3). Although the reason for the higher success rate in HIV-discordant couples than in control couples remains unclear, it is assumed that the females in the HIV-discordant couples, unlike many of those in the control couples, were endocrinologically normal. Furthermore, all of the females and babies remained HIV-1 negative throughout the study period.

Although the number of patient couples treated in this study was smaller that in previously reported studies, we nonetheless were able to establish the safety of the modality. The HIV-1 RNA and proviral DNA were measured twice, just after adjustment of the semen and just before embryo transfer, by the nested PCR method with a detection limit of one copy, for each patient.

In conclusion, the technology employed in this study is considered to offer promising results for HIV-1-discordant couples, allowing those who wish to conceive to do so more effectively and safely. In future, however, it will be necessary to increase the number of patients examined in order to more fully elucidate the safety and efficacy of this technique.

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REFERENCES

1. Semprini, A.E., Levi-Setti, P., Bozzo, M., et al. (1992): Insemination of HIV-negative women with processed semen of HIV-positive partners. Lancet, 340, 1317-1319.
2. Kato, S., Hanabusa, H., Kaneko, S., et al. (2006): Complete removal of HIV-1 RNA and proviral DNA from semen by the swim-up method: assisted reproduction technique using spermatozoa free from HIV-1. AIDS, 20, 967-973.
3. Pena, J.E., Thornton, M.H. and Sauer, M.V. (2003): Assessing the clinical utility of in vitro fertilization with intracytoplasmic sperm injection in human immunodeficiency virus type 1 serodiscordant couples: report of 113 consecutive cycles. Fertil. Steril., 80, 356-362.
4. Araneta, M.R., Mascola, L., Eller, A., et al. (1995): HIV transmission through donor artificial insemination. JAMA, 273, 854-858.
5. Semprini, A.E., Fiore, S. and Pardi, G. (1997): Reproductive counselling for HIV-discordant couples. Lancet, 349, 1401-1402.
6. Marina, S., Marina, F., Alcolea, R., et al. (1998): Human immunodeficiency virus type 1-serodiscordant couples can bear healthy children after undergoing intrauterine insemination. Fertil. Steril., 70, 35-39.
7. Weigel, M.M., Gentili, M. and Beichert, M. (2001): Reproductive assistance to HIV-discordant couples-the German approach. Eur. J. Med. Res., 6, 259-262.
8. Bujan, L., Pasquier, C., Labeyrie, E., et al. (2004): Insemination with isolated and virologically tested spermatozoa is a safe way for human immunodeficiency type 1 virus-serodiscordant couples with an infected male partner to have a child. Fertil. Steril., 82, 857-862.
9. Nicopoullos, J.D., Almeida, P.A., Ramsay, J.W.A., et al. (2004): The effect of human immunodeficiency virus on sperm parameters and the outcome of intrauterine insemination following sperm washing. Hum. Reprod., 19, 2289-2297.
10. Savasi, V., Ferrazzi, E., Lanzani, C., et al. (2007): Safety of sperm washing and ART outcome in 741 HIV-1-serodiscordant couples. Hum. Reprod., 22, 772-777.
11. Zhang, H., Dornadula, G., Beumont, M., et al. (1998): Human immunodeficiency virus type 1 in the semen of men receiving highly active antiretroviral therapy. N. Engl. J. Med., 339, 1803-1809.
12. Marina, S., Marina, F., Alcolea, R., et al. (1998): Pregnancy following intracytoplasmic sperm injection from an HIV-1-seropositive man. Hum. Reprod., 13, 3247-3249.
13. Loutradis, D., Drakakis, P., Kallianidis, K., et al. (2001): Birth of two infants who were seronegative for human immunodeficiency virus type 1 (HIV-1) after intracytoplasmic injection of sperm from HIV-1-seropositive men. Fertil. Steril., 75, 210-212.
14. Sauer, M.V. and Chang, P.L. (2002): Establishing a clinical program for human immunodeficiency virus 1-seropositive men to father seronegative children by means of in vitro fertilization with intracytoplasmic sperm injection. Am. J. Obstet. Gynecol., 186, 627-633.
15. Ohl, J., Partisani, M., Wittemer, C., et al. (2003): Assisted reproduction techniques for HIV serodiscordant couples: 18 months of experience. Hum. Reprod., 18, 1244-1249.
16. Chu, M.C., Pena, J.E., Thornton, M.H., II, et al. (2005): Assessing the treatment efficacy of IVF with intracytoplasmic sperm injection in human immunodeficiency virus-1 (HIV-1) serodiscordant couples. Reprod. Biomed. Online, 10, 130-134.

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A case of neonatal alloimmune thrombocytopenia from human platelet antigen 5b incompatibility

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alloimmune thrombocytopenia, human-specific platelet antigen, amniocentesis, mixed passive hemagglutination

Abstract

Anti-human platelet-specific antigen (HPA) antibody often causes neonatal alloimmune thrombocytopenia (NAIT). The antibody is produced due to the feto-maternal transfusion of incompatible platelets. In this case study, anti-HPA-5b was detected in the serum of a 30-year-old female patient. Using blood or amniotic fluid, the patient' s HPA-5 phenotype was determined to be a+b-, whereas those of the husband, son and fetus were a+b+. From these findings, we concluded that there was an incompatibility of maternal and fetal HPA. Cordocentesis was performed at 34 weeks of gestation and the fetal platelet count was sufficient for vaginal delivery. A transfusion of HPA-matched platelet was prepared. The baby was delivered by vaginal delivery and there were no physical signs of thrombocytopenia.

Case report

A 30-year-old Japanese woman with gravida 2, para 1, gave birth to a female neonate by vaginal birth at 40 weeks of gestation. Two years before, she gave birth to a male neonate with IUGR and congenital duodenal atresia, whose platelet count at the time of birth was 219 $\times 10^9$/L. His platelet count dropped transiently to 79 $\times 10^9$/L on day 14, but returned to 425 $\times 10^9$/L on day 22 without any treatment (Fig. 1). No intracranial hemorrhage, purpura, or petechiae were noted. During this previous pregnancy, the mother's platelet count was 119 $\times 10^9$/L at 38 weeks of gestation and increased

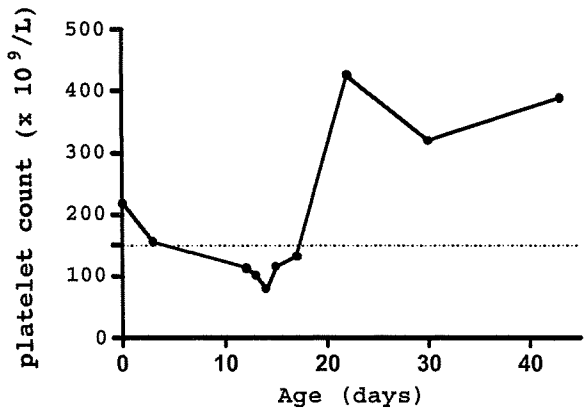

Fig.1 Clinical course of the patient's previous child. Initial platelet count was 219 × 10^9/L. Platelet count at day 14 dropped to 79 × 10^9/L. Without any treatment, platelet count normalized by day 22.

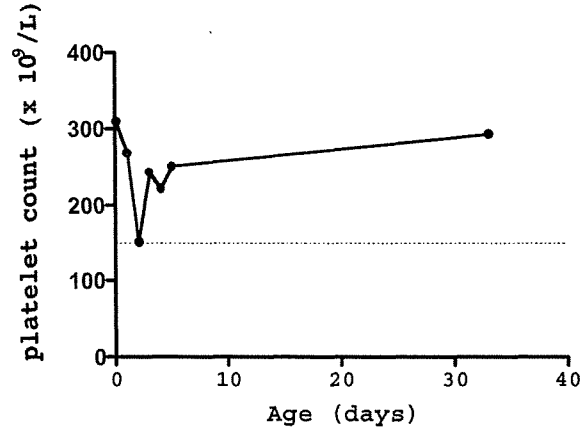

Fig.2 Clinical course of the daughter. Initial platelet count was 310 × 10^9/L. Platelet count at day 2 dropped to 151 × 10^9/L. Without any treatment, platelet count normalized by day 3.

Table 1 HPA type (MPHA method) of the patient, husband,son and fetus

	2b	3a	4a	4b	5a	5b	6b
patient	+	+	+	-	+	-	-
husband	+	-	+	-	+	+	-
son	+	+	+	-	+	+	-
fetus	+	+	+	-	+	+	-

The platelet phenotype of the patient was HPA-5(a+b-) while the husband and children platelet phenotypes were HPA-5(a+b+).

to 217 × 10^9/L on day 14 after delivery.

The patient's platelet count was 140 and 141 × 10^9/L at 8 and 14 weeks of gestation, respectively. A platelet-specific antibody, anti-HPA-5b, was detected with a titer of 1:2048 in her serum, using the mixed passive hemagglutination (MPHA) method, suggesting that the former child's thrombocytopenia might have been due to neonatal alloimmune thrombocytopenia (NAIT). Platelet genotyping of the patient, her husband, and her son gave a result of HPA-5 (a+b-), HPA-5 (a+b+) and HPA-5 (a+b+) (Table 1). Because no blood transfusion was given, the patient could have been sensitized during the previous pregnancy through her son; we diagnosed that the son had neonatal alloimmune thrombocytopenia due to the anti-HPA-5b antibody. Since the fetus also had a 50% risk of NAIT, amniocentesis for fetal genotyping was performed at 28 weeks of gestation. The platelet genotyping of the fetus was HPA-5 (a+b+) using amniotic fluid (Table 1). Since the symptoms of NAIT were more severe for the fetus than for the former child, cordocentesis was performed at 34 weeks of gestation to count the fetal platelet and decide the delivery mode. The platelet count was 203 × 10^9/L and hemoglobin was 117 g/L. Peak systolic velocity in the middle cerebral artery of the fetus was measured up to delivery and no evidence of fetal anemia was noted. After vaginal birth, the platelet count of the female neonate was 310 X 10^9/L. The girl had no purpura or petechiae, so HPA-matched platelet transfusion was not attempted. The platelet count of the neonate dropped to 151 × 10^9/L on day 2, but retuned to 243 × 10^9/L on day 3 without any treatment (Fig. 2). Anti-HPA-5b was detected in the umbilical cord blood.

Discussion

The incidence of anti-HPA antibody is 0.6-0.9% in pregnant women [1, 2].

NAIT occurs when the maternal antibodies of an immunized antigen-negative mother cross the placenta and cause destruction of sensitized fetal platelets [3]. NAIT recurs in 70 to 90 percent of subsequent pregnancies, is often severe, and usually develops earlier with each successive pregnancy [4]. Furthermore, severe thrombocytopenia places the baby at risk for intracranial hemorrhage and other bleeding complications [5].

Due to the former birth of an affected child, an occurrence of NAIT was suspected in this case. Using the MPHA method, anti-HPA-5b was strongly detected with a titer of 1:2048 in the patient's serum. Platelet genotyping of the patient, her husband, and her son gave a result of HPA-5 (a+b-), HPA-5 (a+b+), and HPA-5 (a+b+), confirming that the son's disease was NAIT due to the incompatibility of platelet antigen. The patient received no transfusion, so the HPA-5b antigen could have been sensitized by feto-maternal transfusion during the previous pregnancy. Since the fetus had a 50% risk of NAIT because the husband's genotype was heterozygous, an amniocentesis, which is less invasive, was performed for DNA typing at 28 weeks of gestation after informed consent was obtained. The result was HPA-5(a+b+) and it was diagnosed that there was feto-maternal incompatibility of HPA-5b.

For "standard risk" patients, who are defined as women with documented alloimmune thrombocytopenia who did not deliver an infant with an intracranial hemorrhage in a prior pregnancy [6], it is recommended that vaginal delivery be allowed only for patients whose fetuses have a platelet count greater than 100 × 10^9/L