垂体への照射30 Gy以上(RR：0.61)，卵巣・骨盤への照射5～10 Gy(RR：0.56)，10 Gy以上(RR：0.18)，アルキル化剤のlomustine(RR：0.44)，cyclophosphamide(RR：0.80)と報告している[10]．また，早発閉経をきたす頻度がCCS女性では8%と同胞(0.8%)より高いことを示した[11]．

St Jude Lifetime Cohort Studyでは，年齢中央値32歳(18～60歳)，診断後年数25年(10～47年)の成人CCS 1,713名において，内分泌異常または生殖機能異常を62.0%に認めた[12]．原発性卵巣機能異常(評価項目：月経異常，FSH，estradiol)11.8%，男性胚細胞障害(精液検査)66.4%，Leydig細胞異常(朝のテストステロン，LH)11.5%，と報告されているが，対象集団が若年なため早発閉経を認めていない可能性があり，妊孕性低下について把握されていない．

海外ではがん患者の性腺機能や妊孕性の問題に対する取組みを学会主導で進めている．アメリカ臨床腫瘍学会(American Society of Clinical Oncology)が2013年改訂した診療指針では，がんの治療開始時から妊孕性温存について患者に説明をするようにと述べている[13]．小児がん患者の妊孕性温存に関しては，思春期以後の小児においては確立した妊孕性温存療法(精子凍結保存と卵子凍結保存)を患者あるいは保護者の同意のもとに実施すること，まだ研究段階ではあるが小児で実施可能な他の方法について情報を提示すること，可能であれば実験的手法について問い合わせること，と書かれている．また，アメリカ産科婦人科学会は2014年8月に，がん治療の前後および治療中に女児および未成年の女性のがん患者が抱える婦人科系の悩みを産婦人科医が認識し対処することを求める勧告を発表している．

生殖医療ネットワークの構築

著者らは平成26年度より厚生労働科学研究費補助金がん対策推進総合研究事業(がん政策研究事業)として，“小児・若年がん長期生存者に対する妊孕性のエビデンスと生殖医療ネットワーク構築に関する研究”班(研究代表者：三善陽子)を立ちあげた．小児・若年がん患者の性腺機能・妊孕性の診療にかかわるさまざまな領域の医師(小児腫瘍医，小児内分泌医，産婦人科医，泌尿器科医，生殖医療専門医，精神神経科医など)によるネットワークを構築し，この問題への取組みをはじめている．CCSの性腺機能と妊孕性に関する診療の現状把握を目的として，日本小児内分泌学会の理事と評議員178名を対象として“小児・若年がん患者に対する生殖医療に関するアンケート調査”をCCS委員会の協力のもとで実施した．有効回答数151名(回収率84.8%)で，“性腺機能保持・妊孕性温存に向けて今後求めること(自由記載欄)”に回答者の半数から意見が寄せられ，小児内分泌医の関心の高さが示された．小児がんの治療前に性腺機能低下・妊孕性低下の可能性について説明しているのは主に腫瘍治療医であるが，フォローアップ外来で不妊を告知する辛い役目は小児内分泌医が担っている．生殖医療の技術の進歩に期待する一方で，その不確かさへの不安もあり，幅広い知識と最新情報，ガイドラインを求めていることが示された．

小児における問題点

小児がん患者における大きな問題点は思春期になってから性腺機能異常が表面化することであり，異常に気づかれていない場合もある．長期フォローアップ外来から徐々に足が遠のき，大学進学や就職などを契機に通院が途絶えてしまう．成人後パートナーをもつ時期になってはじめて妊孕性低下という深刻な問題に直面する．低年齢の小児がん患者自身には病名告知や病状説明がなされていない場合も多く，元気に社会復帰した子どもに辛い記憶を思い出させたくないという親の思いから，妊孕性低下の説明はおろか，原疾患や晩期合併症について説明されないままのケースも少なくない．性の知識に乏しく，肉体的・精神的に未熟な小児がん患者はさまざまな問題を抱えている．

わが国においても近年，若年乳がんなどの成人がん患者における治療前の妊孕性温存への配慮が推奨されている[14]．女性患者の場合は卵子を採取する方法があるが，採取にかかる時間と侵襲が問題となる．性的に未熟な小児では卵巣組織の凍結保存が海外で行われつつあるが，がん細胞の浸潤

の可能性がある疾患では適応とならない．わが国では倫理委員会の承認を受けた施設でのみ行われるべき段階で，まだ実施に向けて課題が多い医療である．妊娠・出産時の問題として放射線照射による骨盤・子宮の発育不良，流早産のリスクが報告されているが，次世代への影響(奇形，がん)は遺伝性のがんを除き否定的とされている[11]．

おわりに

がんの治療においては“救命”することが最優先である．妊孕性温存療法のために原疾患の治療がおろそかになることはけっして許されない．安全性の確立していない実験的手法に過剰な期待を抱くべきではない．しかし，治療後の社会生活上のQOL向上を視野に入れた治療戦略が求められている．肉体的・精神的に未熟で自己判断・自己決定が困難な小児がん患者では，医学的・精神心理的・倫理的・法的な問題が山積みであり，とりわけ慎重な対応が望まれる．がん診療と妊孕性温存の両立をめざす“がん・生殖医療(oncofertility)”の発展のために，この問題に対する医師・看護師・心理士など多職種の連携と医療界への広い啓発が必要である．

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トピックス

小児がん患者の性腺機能と妊孕性温存

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1．はじめに

小児がんとは15歳以下の子どもに発生する悪性腫瘍であるが，その種類と治療の多様性が特徴である［1］．小児の病死死因の1位を占めているが，治療プロトコールと支持療法の改良により治療予後が改善し，5年以上生存率は小児がん全体で約8割となっている［2］．小児がん経験者（Childhood Cancer Survivors；CCS）に生じるさまざまな健康障害が近年注目され，長期フォローアップの重要性に対する認識が医療者側に高まっている，一方CCS側のニーズに立てば自らの社会生活に直結する妊娠出産（妊孕性）の問題が重大で，Adolescent and Young Adult（AYA）世代を含む挙児希望の若年がん患者のQOLに大きな影響をもたらしている．

2．国内における小児がん患者の現状

晩期合併症（晩期障害：late effects）は原病による侵襲や治療に起因する直接的または間接的な障害であるが，治療後年数を経てから発症する可能性があるため長期フォローアップが重要である．海外のフォローアップガイドラインとして，Children's Oncology Group Long-Term Follow-Up Guidelines for Survivors of Childhood, Adolescent, and Young Adult Cancers（COG-LTFUガイドライン）が有名である［3］．とりわけ晩期合併症に占める内分泌異常の頻度が高いことから，日本小児内分泌学会CCS委員会により「小児がん経験者（CCS）のための内分泌フォローアップガイドver1.1」が作成され，学会ホームページのガイドラインの項からダウンロード可能である［4］．

小児がんの治療後に性腺機能異常や妊孕性低下が生じることは理解されてきているが，本邦におけるエビデンスは乏しい．筆者らは国内で先駆けて自施設におけるCCSの内分泌学的晩期合併症の現状を報告し，性腺機能低下症の頻度が高いことを示した［5］．CCS122名（男性62名，女性60名）中82名（67%）に内分泌異常を認め，性腺機能低下症を60名（49%）（原発性51名，中枢性9名）と高頻度に認めた．次いでわれわれは成人女性の卵巣予備能の指標とされる抗ミュラー管ホルモン（Anti-Müllerian Hormone：AMH）に着目した．当科通院中の小児152名の血清AMH値について検討し，AMH低値群にCCSが多数含まれることを示し［6］，次いで対象をCCSにしぼった解析によりAMH測定がCCSの卵巣機能評価に有用であることを示した［7］．CCS女性の53%がAMH低値を示したのに対し，FSH高値は30%のみで，移植後患者のAMHは著明に低下していた．長期フォローアップガイドライン［3，4］では月経，血中ゴナドトロピンと性ホルモンが評価項目とされているが，FSH高値を示さない思春期発来前の女児や月経を認めるサバイバー女性において卵巣機能異常が見逃される可能性を示した．

3．海外における小児・若年がん患者の現状

海外では複数の大規模コホート研究が行われている．有名な北米のChildhood Cancer Survivor study（CCS study）では，1970-86年にがんと診断された10397名を同胞と比較した調査において晩期合併症が年々増加することを明らかにした［8］．彼らはこのコホート集団を用いてさまざまな健康障害の解析を行っており，CCS女性は同胞に比べて妊娠率が0.81と低下し，早発閉経の頻度（8%）が同胞（0.8%）より高率であることを示した［9，10］．妊娠・出産時の問題として，アルキル化剤による心毒性，放射線照射による骨盤・子宮の発育不良，流早産のリスクが報告されている．

本邦でも若年乳がん患者における妊孕性温存への配慮が近年推奨されるようになってきたが［11］，海外では学会主導でがん患者の性腺機能や妊孕性に対する取り組みを進めている．米国臨床腫瘍学会（American Society of Clinical Oncology）が2013年改訂した診療指針（ASCO 2013）では，がんの治療開始時から妊孕性温存について患者に説明するようにと述べている［12］．小児がん患

者に関しては，思春期以後の小児において確立した妊孕性温存療法（精子と卵子の凍結保存）を患者もしくは保護者の同意の下に実施すること，まだ研究段階ではあるが小児で実施可能な他の方法について情報を提示することなどが記載されている．また米国産科婦人科学会は2014年8月，癌の治療前後および治療中の女児や未成年の女性がん患者が抱える婦人科系の悩みを産婦人科医が認識し対処することを求める勧告を発表している．

4．生殖医療ネットワーク構築

われわれは平成26年度より厚生労働科学研究費補助金がん対策推進総合研究事業(がん政策研究事業)として，「小児・若年がん長期生存者に対する妊孕性のエビデンスと生殖医療ネットワーク構築に関する研究」班（研究代表者：三善陽子）を立ち上げた．小児・若年がん患者の性腺機能・妊孕性の診療に関わるさまざまな領域の医師（小児腫瘍医，小児内分泌医，産婦人科医，泌尿器科医，生殖医療専門医，腫瘍内科医，精神神経科医など）によるネットワークをまず構築した．初年度にはCCSの性腺機能と妊孕性に関する診療の現状把握を目的として，日本小児内分泌学会の理事と評議員178名を対象として，「小児・若年がん患者に対する生殖医療に関するアンケート調査」を小児内分泌学会CCS委員会の協力のもとに実施した[13]．有効回答数は151名（回収率84.8%）で，「性腺機能保持・妊孕性温存に向けて今後求めること（自由記載欄）」に対しても多くの医師が意見を記載し，小児内分泌医の関心の高さが示された．がんの治療前に性腺機能低下・妊孕性低下のリスクについて説明するのは主に腫瘍治療医であるが，フォローアップ外来で実際にこの問題に対応しているのは内分泌医や生殖医療医であり，自由記載欄では医師の連携体制が必要という意見が最も多く寄せられた．正確な情報提供，腫瘍医や内分泌医への情報フィードバック，看護師やカウンセラーなど多職種との連携などを求めていた．

そこでわれわれは多職種による情報提供と意見交換の場として，「がんと生殖に関するシンポジウム2015～小児・若年がん患者さんの妊孕性温存について考える～」や，がん医療従事者向け研修会「がんサバイバーシップを科学する～がんと生殖医療～」を開催した．本研究班ではこの他にも，ポータルサイトの開設，CCS自身へのアンケート調査，がん患者の妊孕性に対する医師の意識調査，CCSの妊娠・出産の実態調査，CCS女性の性腺機能・妊孕性に関する多施設前向きコホート研究，相談窓口の整備，若年早期乳癌患者に対する生殖技術の安全性および治療後の妊孕性に関するデータベース構築のためのパイロット研究，若年がん患者の妊孕性温存を目的とした未熟精巣組織凍結保存法の確立に関する研究などにも取り組んでいる．

5．おわりに

小児がん患者は治療自体が無事終了しても，思春期になってから性腺機能異常が明らかとなり，成人になってから妊孕性低下に直面する可能性がある．晩期合併症の説明はおろか病名すら本人に告知されていない場合もあり，長期フォローアップの必要性を認識しないことによる「受診の中断」をもたらし，成人後に初めて不妊の説明を受けることが問題となっている．がんの治療は「救命」することが最優先であるが，治療後の社会生活上のQOL向上を視野に入れた治療戦略も求められている．がん診療と妊孕性温存の両立をめざす「がん・生殖医療(Oncofertility)」の発展のために，診療科の枠を越えた取り組みと医療界への啓発が必要である．

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産科婦人科疾患最新の治療 2016-2018

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7 Oncofertility：がん治療と生殖医療

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近年のがん治療において，がん患者の生存率は飛躍的に向上している．しかし，化学療法や放射線療法は，卵巣予備能を破綻させ不妊を誘導する．したがって生殖腺毒性を伴う治療が必要となる患者には，妊孕性の温存という選択肢を示すことが重要となってくる．なぜなら，自身の子どもを産めるということが，後のQOLにおいて重要な一因となり得るからである．そして近年では，生殖腺毒性を伴う治療が必要となるがん患者でも，それぞれの状況に応じた妊孕性の温存治療を選択でき，自身の受精卵を用いて妊娠・出産が可能となってきた．たとえば，卵子凍結保存，胚凍結保存はすでに十分確立された技術といえる[1,2]．このどちらを選択するかはさまざまな要因に依存しており，がん患者であれば，本人の年齢やパートナーの状況，がんのタイプ・進行度などが関わってくる．

さらに，最近になって，卵巣組織の凍結保存という，新たな選択肢が選択可能になった．この卵巣組織の凍結保存は，今後選択者の増加が予想される妊孕性温存技術として，さらなる改良が加えられている．なぜなら，凍結保存した卵巣組織を移植することで複数の卵子が獲得できる可能性を残せるという妊孕性温存の観点だけでなく，ホルモン不足に由来するQOLの低下を防ぐことができるからである[3]．

本項では特に女性のがん患者に対して，がん治療が及ぼす生殖能への危険性とともに，現在選択できる妊孕性温存のための生殖医療について概説する．

がん治療と妊孕性

現在，女性におけるがん患者の10％程度が45歳以下に発症すると考えられている．そのなかで，化学療法および放射線療法や骨髄移植などの治療を必要とする女児や若年女性の90％以上が，これらの治療を行うことで疾患を克服している[4]．しかしながら，卵巣は抗がん剤などの細胞毒性薬（特にアルキル化薬）に対し非常に敏感であり，これらの薬剤は生殖腺機能に障害を与える危険性を有するものに分類されている[5,6]．なかでも，シクロホスファミドは濃度依存的に卵子や顆粒膜細胞に対するダメージに関わる物質として広く知られている[5,6]．腹部に対する放射線療法と，それに付随するアルキル化薬の化学療法は時に早発卵巣不全（premature ovarian insufficiency：POI）を引き起こし，そのほとんどで患者は不妊となる．実際に放射線量5～20 Gyの卵巣に対する照射によって，生殖腺機能が完全に消失するという報告もある[5-7]．また，骨髄移植に必要な強化化学療法，もしくは放射線の全身照射によるPOI発症の危険性は極めて高いとされ，その危険性は約90％と報告されている[8]．他に，アルキル化薬であるブスルファンを用いた化学療法からの骨髄移植を受けた場合，卵巣機能の回復見込みはほとんどないとの報告もある[9]．このように，種々抗がん剤などの化学療法もしくは放射線療法を伴う治療は，女性の生殖腺機能に大きな影響を与えることが明らかになっている．一方で，これらの治療に耐えた若年女性の多くが，その疾患を克服できていることも忘れてはならない．

妊孕性を温存するための生殖医療

配偶子および生殖組織の保存を含む妊孕性の温存は生殖医療分野において，急速に進歩してきた．この技術は，手術もしくは化学療法や放射線療法を受けるがん患者をはじめとして，POIに通ずるその他の疾患を抱える女性，妊娠を延期したい健常女性にとっても大変有益なものとなっている．卵子，胚もしくは卵巣組織の凍結保存は，妊孕性温存の選択肢の1つである．

1 卵子凍結，胚凍結

哺乳動物における胚の凍結保存は，1952年にウサギを用いた研究でその可能性が示された[10]．その後，動物実験を経て，1983年に緩慢凍結法を用いて凍結保存した胚を移植することで，ヒトで初めての妊娠例が報告されている[11]．一方，卵子の凍結保存は，1986年に同じく緩慢凍結法により，ヒトで初めて妊娠例が報告された[12]．しかし，融解後の卵子の生存率は約60％と低率であり，その体外受精の成績も新鮮卵子のそれに及ばなかった．その後，2000年頃までは，この緩慢凍結法が生殖補助医療における胚凍結保存法の主流であったが，この方法には高確率な細胞質内の氷晶形成，長時間低温にさ

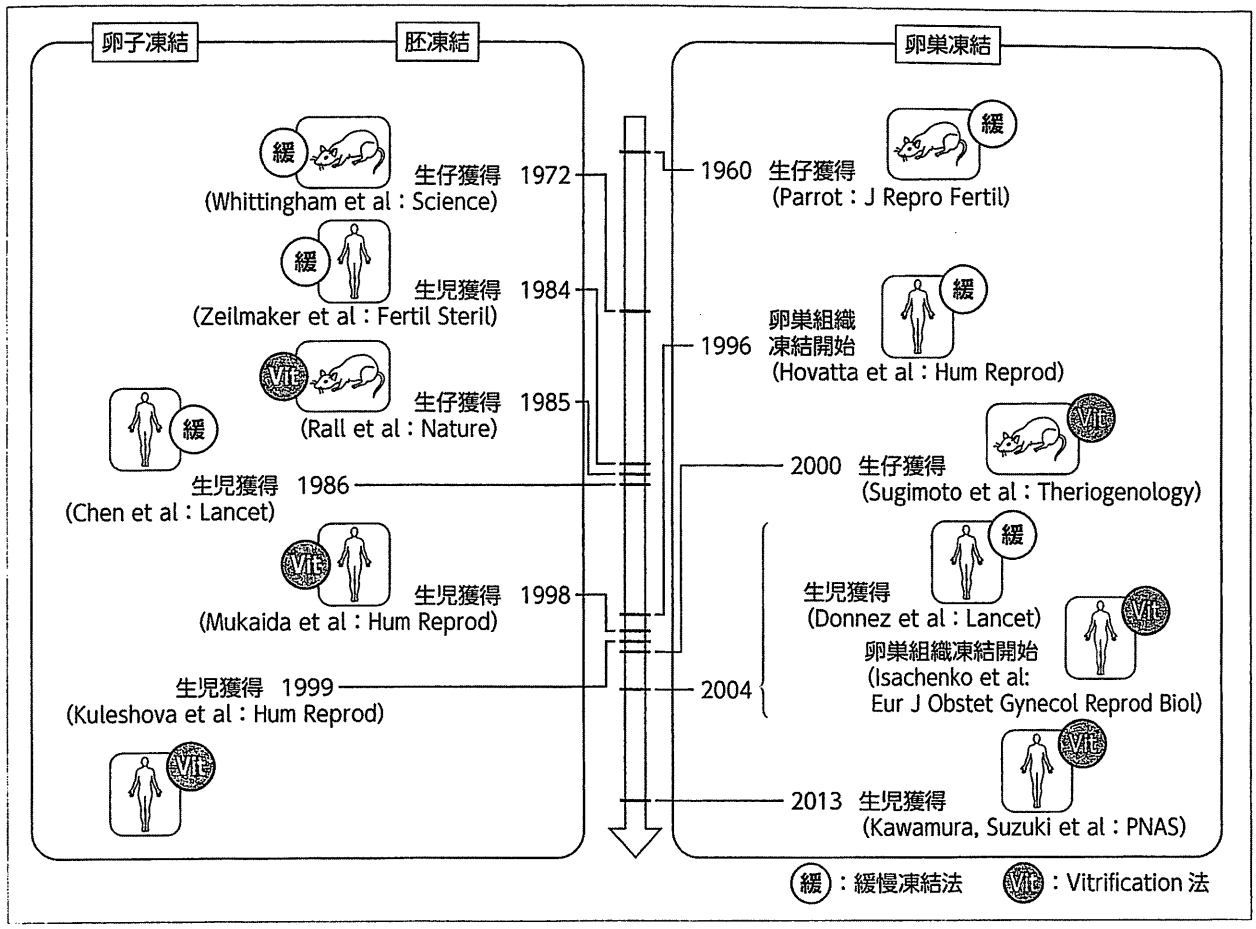

図1 卵子および胚凍結と卵巣組織凍結保存の歴史

らすことでの冷害や浸透圧変化による障害などの改善すべき点が残っていた.

これらを改善し，現在の卵子および胚凍結保存の主流となったのが vitrification 法である. vitrification 法は，1985 年にマウス胚を用いた研究で産仔獲得が初めて報告され[13]，2000 年には臨床応用可能な至適条件が報告された[14]. これにより卵子および胚の凍結融解は，簡便かつ短時間に実施可能となり，生存率などの臨床成績も向上した．特に卵子凍結においては，低率であった融解後の生存率，その後の体外受精成績が新鮮卵子と比較しても遜色ないところまで改善された．しかしながら，その体外受精の成績も，凍結融解卵 1 個あたりの妊娠率は 4.5～12%であり，凍結融解胚（35 歳未満の胚盤胞の場合）と比べると未だ良好とはいえないのが現状である.

卵子もしくは胚凍結を行う場合，後の妊娠・出産を考えると 1 つでも多くの卵子もしくは胚を保存しておくのが理想的である．しかしながら，がんの発見から治療開始までの時間は多くの場合限られており，診断から治療開始までの期間が長く伸びるほど予後不良とされている[15]. 必然的に短期間でより多くの卵子獲得を試みることになり，FSH および hCG による過排卵刺激が適応される．しかし，がんの種類によっては，過排卵刺激に際し増悪または再発のリスクを伴うものも報告されており，若年女性におけるがん罹患数上位にある乳がんはその代表である. 近年では，乳がん患者に対して妊孕性の温存治療目的で過排卵刺激を行う際は，前述リスク要因の 1 つであるエストロゲン合成を阻害するアロマターゼ阻害薬の併用が注目されている[16]. このように，今まで原疾患との兼ね合いから妊孕性の温存が困難とされてきたケースでも，それを可能にする方法が見出されており，これらの研究は今後さらに進められていくと期待される.

2 卵巣組織凍結

卵巣組織はそのときの月経周期に関係なく腹腔鏡手術で比較的容易に採取できるため，前述した卵子および胚保存のように化学療法や放射線療法の遅延を懸念することもない．この方法は卵巣内により多くの卵子が残っている 35 歳

以下の若年がん患者に適しており，摘出した卵巣は生殖医療の専門機関で凍結保存でき，がん治療によって患者自身がPOIになってしまった場合でも，移植が可能である．したがって，多くの卵胞が生存可能となった凍結融解法が確立されて以来，がん治療前の卵巣組織凍結保存は優れた妊孕性温存技術となった[17]．

卵巣組織凍結保存の歴史は1954年に遡る．その後，卵子・胚凍結保存同様，緩慢凍結法およびvitrification法の双方が実験動物を経てヒトに応用されている（図1）．2004年にはヒトで初めて，緩慢凍結法を用い凍結保存した卵巣組織を融解後，自家移植し，妊娠および出産に至った例が報告されている[18]．この卵巣組織凍結保存技術を用いて少なくとも60人の生児獲得が報告されているが[19]，近い将来，この数はさらに増加することが予想される．なぜならば，vitrification法を用いた卵巣組織凍結が臨床で用いられ始めたのは最近のことであり，2013年になってヒトで初めて出産例が報告されたが[20]，そのほとんどは未だ凍結保存中であり，移植後の成績は未知数なためである．

卵巣組織凍結保存における緩慢凍結法とvitrification法の比較についても，近年複数の報告が出ているが，前述した理由もあり組織学的な解析にとどまっており，現状では双方の保存法にはっきりとした差はないものといえる．また，卵巣組織凍結保存において白血病などは，血中に遊走するがん細胞が凍結保存した卵巣組織に残存し移植後の再発リスクを伴うという理由から適応外となっている．卵巣組織凍結保存，およびその再移植技術は未だに研究段階とされているにもかかわらず，多数の生児獲得の報告がなされている今，欧州で考えられているように，この技術はすでに確固たる域に達しているとも考えられる．しかしながら，安全性と有効性に関して今後さらなる研究ならびに改良が進められ，適応外となっている疾患の患者に対しても，この技術が適応される時期が来ることが期待される．

妊孕性温存技術は今後さらに進歩を続けるものと予想される．これは妊孕性の温存を希望する患者にとって，その選択肢を広げ，治療後のQOL向上にも直結するものである．加えて，原疾患の治療に専念できる環境作りにも貢献する可能性が考えられる．今後必要となってくるのは，言うまでもなく，生殖腺毒性を伴う治療を必要とする患者すべてに不妊の危険性，妊孕性を温存する技術に関する情報を与えることである．そしてそれを可能にするため，日々進化する妊孕性温存技術の最新動向を常に把握していくことが重要になると考える．

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human reproduction ORIGINAL ARTICLE *Infertility*

Successful fertility preservation following ovarian tissue vitrification in patients with primary ovarian insufficiency

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STUDY QUESTION: Is ovarian tissue cryopreservation using vitrification followed by *in vitro* activation (IVA) of dormant follicles a potential approach for infertility treatment of patients with primary ovarian insufficiency (POI)?

SUMMARY ANSWER: Our vitrification approach followed by IVA treatment is a potential infertility therapy for POI patients whose ovaries contain residual follicles.

WHAT IS KNOWN ALREADY: Akt (protein kinase B) stimulators [PTEN (phosphatase with TENsin homology deleted in chromosome 10) inhibitor and phosphatidyinositol-3-kinase (PI3 kinase) stimulator] activate dormant primordial follicles *in vitro* and ovarian fragmentation disrupts the Hippo signaling pathway, leading to the promotion of follicle growth. We treated POI patients with a combination of ovarian vitrification, fragmentation and drug treatment, followed by auto-transplantation, and reported successful follicle growth and pregnancies.

STUDY DESIGN, SIZE, DURATION: Prospective clinical study of 37 infertile women with POI between 12 August 2011 and 1 November 2013. We enrolled 10 new patients since the previous publication.

PARTICIPANTS/MATERIALS, SETTING, METHODS: POI patients were originally selected based on a history of amenorrhea for more than 1 year and elevated serum FSH levels of >40 mIU/ml ($n = 31$) but this was later changed to >4 months, age <40 years and serum FSH levels of >35 mIU/ml ($n = 6$) (mean 71.8 ± 30.8, range 35.5–197.6) so as to include patients with a shorter duration of amenorrhea. Under laparoscopic surgery, ovariectomy was performed and ovarian cortices were dissected into strips for vitrification. Some pieces were examined histologically. After warming, two to three strips were fragmented into smaller cubes before culturing with Akt stimulators for 2 days. After washing, ovarian cubes were transplanted beneath the serosa of Fallopian tubes under laparoscopic surgery. Follicle growth was monitored by ultrasound and serum estrogen levels. After oocyte retrieval from mature follicles, IVF was performed.

MAIN RESULTS AND THE ROLE OF CHANCE: Among 37 patients, 54% had residual follicles based on histology. Among patients with follicles, 9 out of 20 showed follicle growth in auto-grafts with 24 oocytes retrieved from six patients. Following IVF and embryo transfer into four patients, three pregnancies were detected based on serum hCG, followed by one miscarriage and two successful deliveries. For predicting IVA success, we found that routine histological analyses of ovarian cortices and shorter duration from initial POI diagnosis to ovariectomy are valid parameters.

LIMITATIONS, REASONS FOR CAUTION: Although our findings suggest that the present vitrification protocol is effective for ovarian tissue cryopreservation, we have not compared the potential of vitrification and slow freezing in follicle growth after grafting. We chose the serosa of Fallopian tubes as the auto-grating site due to its high vascularity and the ease to monitor follicle growth. Future studies are needed to evaluate the best auto-grafting sites for ovarian tissues. Also, future studies are needed to identify biological markers to indicate the presence of residual follicles in POI to predict IVA treatment outcome.

WIDER IMPLICATIONS OF THE FINDINGS: In POI patients, ovarian reserve, namely the pool of residual follicles, continues to diminish with age. If one ovary is cryopreserved at an earlier stage of POI, patients could undergo additional non-invasive infertility treatments before the final decision for the IVA treatment. Furthermore, in the cases of unmarried POI patients, cryopreservation of ovarian tissues allows their fertility preservation until they desire to bear children.

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Introduction

Ovarian tissue cryopreservation followed by auto-transplantation is a promising method for fertility preservation in women undergoing gonadotoxic treatments (von Wolff *et al.*, 2009; Donnez and Dolmans, 2010; Kolp and Hubayter, 2011; Andersen *et al.*, 2012; Grynberg *et al.*, 2012; Jeong *et al.*, 2012; Gamzatova *et al.*, 2014). This procedure could also be useful for patients suffering from progressive ovarian dysfunction. In contrast to oocyte freezing that requires the storage of mature oocytes obtained from pre-ovulatory follicles, ovarian tissue cryopreservation is suitable for prepubertal girls by preserving their immature oocytes in early ovarian follicles. Following ovarian transplantation and follicle growth, early follicles could develop into pre-ovulatory follicles to allow the generation of mature eggs for fertility treatment. Ovarian tissue cryopreservation also has the advantage of storing a large number of immature oocytes inside early follicles. After the first successful live birth from a patient with Hodgkin's lymphoma following orthotropic transplantation of cryopreserved ovarian tissues (Donnez *et al.*, 2004), several reproduction centers have reported success in restoring fertility in cancer survivors, resulting in live births (Meirow *et al.*, 2005). In all these cases, slow freezing protocols were used for the cryopreservation of ovarian tissues (Donnez *et al.*, 2004).

Following the earlier use of slow freezing for tissue preservation, rapid advances in the vitrification approach have led to successful cryopreservation of preimplantation embryos and mature oocytes (Katayama *et al.*, 2003; Kuwayama *et al.*, 2005; Kuwayama, 2007). The advantages of the vitrification approach include no ice crystal formation that is known to cause physical and mechanical injury of cells. In addition, the vitrification procedure is attractive, because it is a quick and easy way to cryopreserve ovarian tissues and does not require special and expensive equipment. Many studies comparing slow freezing and vitrification of ovarian tissues have been conducted [reviewed in Amorim *et al.* (2011)]. However, the use of divergent vitrification protocols led to conflicting outcomes. Thus, it is becoming important to establish optimal vitrification protocols for the cryopreservation of ovarian tissues for infertility treatments.

Ovarian functions decrease with age, characterized by a diminishing number of follicles and menstrual cycle cessation. In patients with primary ovarian insufficiency (POI), early exhaustion of ovarian follicles is evident due to genetic, immunological, iatrogenic or other causes. POI affects 1% of women and is characterized by high circulating FSH levels together with amenorrhea before 40 years of age (Nelson, 2009). These patients are infertile due to a lack of follicle growth and ovulation; oocyte donation is the only treatment option. Although menstrual cycles cease in these patients, some of them still contain residual small ovarian follicles not producing enough circulating estrogens and progesterone to modulate uterine functions (De Vos *et al.*, 2010).

Our earlier report demonstrated the ability of PTEN (phosphatase with TENsin homology deleted in chromosome 10) inhibitors and phosphatidyinositol-3-kinase (PI3 kinase) stimulators to activate dormant murine and human primordial follicles *in vitro* (Li *et al.*, 2010). We further demonstrated that ovarian fragmentation disrupts the Hippo signaling pathway in the ovary, leading to increased production of downstream CCN growth factors and the promotion of follicle growth (Kawamura *et al.*, 2013). We combined ovarian cryopreservation, fragmentation and *in vitro* activation (IVA) drug treatment (the PTEN inhibitor and the PI3K activator), followed by auto-transplantation, as infertility treatment for POI patients and reported successful follicle growth and pregnancies (Kawamura *et al.*, 2013).

Here, we provide detailed clinical information on our POI patients and protocols of ovarian tissue cryopreservation using vitrification for the successful generation of mature oocytes in POI patients as a potential approach for infertility treatment and update the outcome of an expanded clinical study. We also demonstrated that histological analyses of ovarian cortices after ovariectomy and patient's history of shorter durations from POI diagnosis to ovariectomy are reliable parameters to predict the success of IVA.

Materials and Methods

Patients

For present clinical studies, we obtained informed consent from patients and approval from the Human Subject Committee of St. Marianna University and Japan Society of Obstetrics and Gynecology.

POI patients were originally selected based on a history of amenorrhea for more than 1 year and elevated serum FSH levels of >40 mIU/ml ($n = 31$) but these criteria were later changed to amenorrhea for more than 4 months, age <40 years and serum FSH levels of >35 mIU/ml ($n = 6$; mean 71.8 ± 30.8, range 35.5–197.6) so as to include patients with a shorter duration of amenorrhea. For measurement of anti-Müllerian hormone (AMH) levels, Active MIA/AMH EIA kit or AMH Gen II ELISA kit (Beckman Coulter, Brea, CA, USA) was used according to the manufacturer's protocol. Because the assay for AMH has been changed from Active MIA/AMH EIA to AMH Gen II ELISA, Data obtained by Active MIA/AMH

EIA in earlier patients were converted into the unit (ng/ml) for AMH Gen II ELISA with a multiplication factor of 0.14. The detection limit of AMH Gen II ELISA and Active MIA/AMH EIA were 0.16 ng/ml and 1.0 pmol/l, respectively. Presence of endometriosis was determined based on the pelvic observation during laparoscopic surgery for ovariectomy (Re-AFS: stage I-III). A total of 37 patients were enrolled and received ovariectomy for ovarian tissue cryopreservation, followed by IVA for infertility treatment (Supplementary data, Table SI).

Ovarian tissue cryopreservation

Under laparoscopic surgery, ovariectomy was performed to remove one or both ovaries without using electrocautery hemostasis to avoid damage to residual follicles. After removal, ovaries were immediately cut in half to expose the medulla (Fig. 1A, left panel). The ovary was immersed in modified-HTF medium supplemented with 10% Serum Substitute Supplement (SSS; IrvineScientific, Santa Anna, CA, USA) on a warm plate at 37°C. The majority of small residual follicles were located within a 1–2 mm thickness from the surface of the ovarian cortex in POI patients (Kagawa *et al.*, 2009). Therefore, the medulla was removed by dissection with small scissors before thin layers of ovarian cortices (1–2 mm thickness) were prepared (Fig. 1A, middle panel) and cut into small strips (0.5–1 × 0.5–1 cm, 1–2 mm thickness; Fig. 1A, right panel). This step maximized the surface to mass ratio and the strips were suitable for loading onto the stainless needles of the Cryosupport (Fig. 1B, left panel). For cryopreservation of ovarian tissues, a vitrification method was used as previously described with modifications (Hashimoto *et al.*, 2010; Suzuki *et al.*, 2012). Ovarian strips were washed in TCM199 medium (Life Technologies, Foster City, CA, USA) supplemented with 20% (v/v) SSS (WS medium) and then sequentially exposed to three different vitrification solutions at room temperature. The strips were first equilibrated in TCM199 medium containing 10% (v/v) ethylene glycol (EG, Wako Pure Chemical Industries, Tokyo, Japan) and 20% (v/v) SSS for 5 min, followed by equilibration in the TCM199 medium containing 20% (v/v) EG and 20% (v/v) SSS for 5 min. Subsequently, strips were transferred into the TCM199 medium containing 35% (v/v) EG, 5% (w/v) polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, MO, USA), and 0.5 mol/l sucrose (Wako Pure Chemical Industries) for equilibration before the final step of vitrification. Within 15 min, ovarian strips were loaded on the Cryosupport (Fig. 1) consisting of four fine stainless needles and a cryogenic vial (BD Bioscience, San Jose, CA, USA) before direct immersion vertically into liquid nitrogen with a minimum volume of media. After putting the frozen strips individually into cryogenic vials, ovarian tissues were stored in liquid nitrogen until use. After successful vitrification, cortical strips became transparent in appearance (Fig. 1C, upper device), whereas failed vitrification was characterized by the crystalline appearance of a white cryohydrate (white arrow, Fig. 1C, lower device).

For tissue warming, ovarian strips loaded on the Cryosupport were immersed in 5 ml of TCM199 medium containing 20% SSS and 0.8 mol/l sucrose before warming at 37°C for 1 min. To remove cryoprotectant, ovarian strips were incubated in TCM199 containing 20% SSS and 0.4 mol/l sucrose for 3 min, followed by incubation in the WS medium for 5 min twice at room temperature. Warmed strips were kept in modified-HTF medium supplemented with 10% SSS until the next step.

Videos demonstration of the ovarian tissue preparation, vitrification and warming steps are shown in Supplementary Materials.

Histological analyses of residual follicles in ovarian cortices

To identify residual follicles in ovarian cortices of POI patients, 10–20% of the volume of the cortices was subjected to histological analysis. Pieces of ovarian cortex were fixed in the Bouin's solution for 2 h, embedded in paraffin, serially sectioned at a thickness of 4 μm and then stained with hematoxylin and eosin (H&E). Follicles were detected under a light microscope (Olympus, Tokyo, Japan). If we could detect some follicles in sections, we terminated

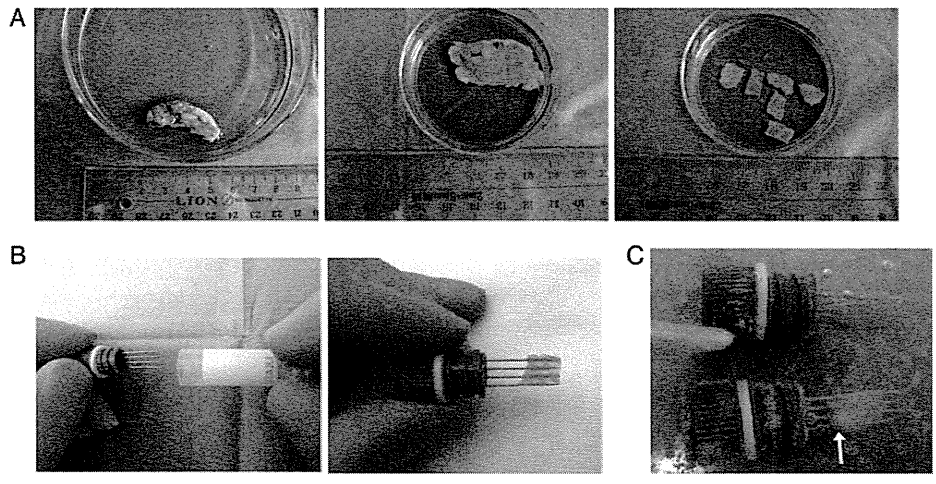

Figure 1 Vitrification of ovarian tissues from POI patients. After ovariectomy under laparoscopic surgery, ovarian cortices were dissected into small strips (0.5–1 × 0.5–1 cm) for vitrification using CryoSupport. (**A**) Preparation of ovarian cortical tissues for vitrification. Representative images were obtained from a patient with POI. Left panel: an ovary before dissection of medulla; middle panel: an ovary after dissection of medulla; right panel: small ovarian strips ready for vitrification. (**B**) The 'CryoSupport' device used for vitrification. Left panel: the CryoSupport composed of four stainless needles inserted into the cap of a cryogenic vial; right panel: an ovarian cortical strip from a POI patient placed on the CryoSupport. Owing to its thin thickness (1–2 mm), the cortical strip appeared transparent. (**C**) Appearance of ovarian cortical strips of POI patients after the initial vitrification procedure. Upper device: successful vitrification is characterized by the transparent appearance of a cortical strip; lower device: failed vitrification is characterized by the crystalline appearance of the white cryohydrate (arrow).

the analyses without examining remaining sections. Absence of residual follicles was decided after observation of all sections.

IVA: ovarian fragmentation, Akt stimulation and auto-transplantation

After patients recovered from the first surgery, two to three pieces of cryopreserved ovarian strips were warmed at 2 days before the second laparoscopic procedure. Ovarian strips were further fragmented into smaller cubes (1–2 mm^3) using a fine scalpel. Six to nine ovarian cubes were put on cell culture inserts (Millicell Cell Culture Insert, 12 mm, polycarbonate, 3.0 μm; Merck Millipore, Darmstadt, Germany). They were treated with 30 μM of bpV(hopic), a PTEN enzyme inhibitor (Merck Millipore), and 150 μg/ml of 740YP (Tocris, Bristol, UK), a PI3 K stimulator, for 24 h followed by incubation with 740YP alone for another 24 h in Dulbecco's Minimum Essential Medium/F12 medium containing 10% (v/v) human serum albumin (Mitsubishi Tanabe Pharma, Tokyo, Japan), 0.05 mg/ml ascorbic acid, 1% (v/v) antibiotic/antimycotic solution (Life Technologies) and 0.3 IU/ml recombinant FSH (GONAL-f; Merck Serono, Tokyo, Japan) at 37°C under a 5% CO_2 atmosphere (Hashimoto *et al.*, 2010). Bioactivities of the PTEN inhibitor and PI3K activating peptide have been pretested using murine models as previously described (Li *et al.*, 2010). For auto-transplantation, ovarian cubes were repeatedly washed for three times in warmed (37°C) culture media immediately before transplantation. Transplantation of ovarian cubes beneath the serosa of one or both Fallopian tubes was performed under the second laparoscopic surgery. The underside of serosa in Fallopian tubes was selected as the grafting site due to high vascularization, convenience for transvaginal ultrasound monitoring, and ease for oocyte retrieval from pre-ovulatory follicles. After cutting the serosa and making a pouch between serosa and Fallopian tube, ~20–100 ovarian cubes were inserted (Fig. 2A, upper and middle panels) followed by closure of the serosa using sutures (Kawamura *et al.*, 2013). Alternatively, the wound was covered by an oxidized regeneration cellulose (Interceed; Johnson & Johnson, Tokyo, Japan) to avoid cube loss from the graft site (Fig. 2A, lower panel).

Monitoring of follicle growth and IVF-embryo transfer

After auto-transplantation of ovarian cubes beneath the serosa of the Fallopian tubes, follicle growth in grafted sites was monitored weekly or biweekly by transvaginal ultrasound together with serum estrogen and gonadotrophin levels to detect growing antral follicles. It has been reported that suppression of endogenous gonadotrophins is important in the induction of folliculogenesis and ovulation in patients with POI likely by restoring the responsiveness of residual follicles to exogenous gonadotrophin stimulation and by mitigating the desensitization to gonadotrophins (Menon *et al.*, 1983; Nelson *et al.*, 1994; Ishizuka *et al.*, 1997). We treated patients with 0.625–1.875 mg estrogen (Premarin; Pfizer Inc.) to suppress elevated endogenous gonadotrophins before exogenous gonadotrophin stimulation. When antral follicles were detected, monitoring frequency increased to every 2–3 days and follicle growth was promoted by injecting 150–300 IU recombinant FSH (Merck Serono) daily until the follicle reached >16 mm in diameter or serum estradiol levels elevated to >200 pg/ml. In some cases, 0.25 mg of a GnRH antagonist, cetrorelix acetate (Cetrotide, Merck Serono), was administrated daily when the growing follicle reached ~14 mm in diameter to avoid the premature LH surge. When follicle reached >16 mm, oocyte maturation was induced by a single injection of 10 000 IU hCG (Gonatropin; Asuka Pharma). A few patients received hCG when follicles reached 13–15 mm due to their personal scheduling reasons. At 36 h later, oocytes were aspirated from follicles using ultrasound-guided, transvaginal retrieval via a 19–20 G needle. After oocyte retrieval, IVF was performed by using ICSI before culturing injected oocytes in the fertilization medium (Quinn's Advantage® Fertilization HTF Universal Medium; CooperSurgical, Trumbull, CT) for 16 h. ICSI was used to increase fertilization success.

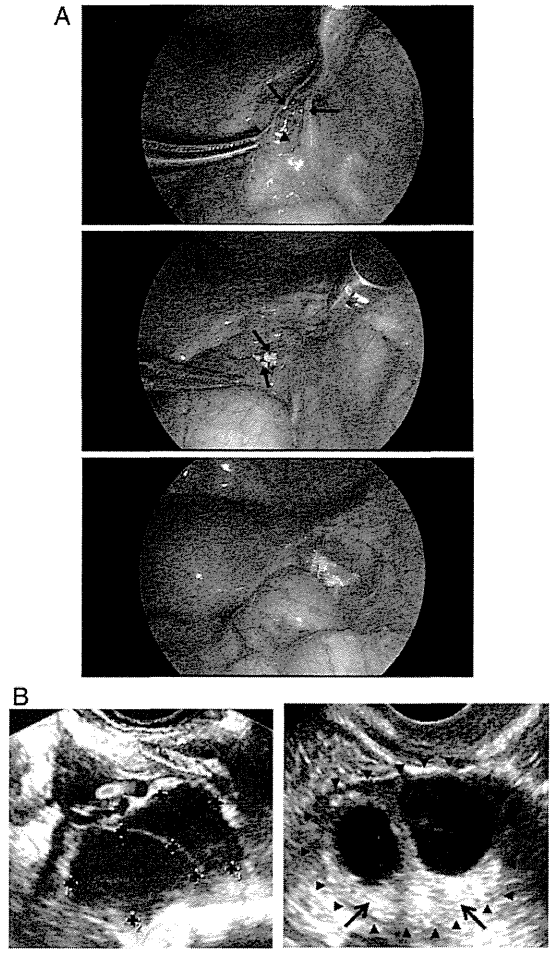

Figure 2 Auto-transplantation of ovarian fragments under laparoscopic surgery and monitoring of follicle growth after grafting. (**A**) Auto-transplantation of ovarian fragments beneath the serosa of a Fallopian tube in a patient with POI. Upper panel: cutting the serosa and making a pouch between serosa (arrows) and Fallopian tube (arrowhead); middle panel: grafting multiple ovarian cubes (arrows) beneath the serosa of Fallopian tubes; lower panel: wound was covered by an oxidized regeneration cellulose to avoid cube loss from the graft site. (**B**) Representative ultrasound images of growing follicles. Left panel: two follicles growing inside grafted ovarian fragments (plus symbols) beneath the serosa of a Fallopian tube in a POI patient. The follicle image is characterized by the absence of neighboring medulla; right panel; two follicles growing inside the ovary in an infertile patient following controlled ovarian stimulation undergoing oocyte retrieval for IVF. The follicle image is characterized by the presence of medullar tissue adjacent to the growing follicle (arrows) and a clear outline of the ovary (arrowheads).

Fertilized oocytes were transferred to the cleavage medium (Global®, LifeGrobal, Tokyo, Japan) and cultured for one more day. Early preimplantation embryos at Day 2 of culture were cryopreserved by vitrification using

Cryotop (Kitazato BioParma; Kuwayama, 2007) and stored in liquid nitrogen until use. To improve embryo implantation of POI patients, embryos were cryopreserved and then transferred following hormone supplementation of patients with estrogens (Premarin and Estrana TAPE; Hisamitsu Pharmaceutical) and then progesterone (Progestone; Fuji Pharmaceutical). The dosage and duration of estrogen and progesterone treatments were determined based on the uterine endometrial status of individual patients. After embryo transfer, patients were treated with 10 mg of oral progesterone daily (Provera; Pfizer Inc.) and 125 mg of i.m. injection of progesterone weekly (Proge depot; Mochida Pharmaceutical) for 6 weeks to support early pregnancy. Establishment of pregnancy was determined by ultrasound and by measuring serum hCG levels. After the patient became pregnant, development of fetus was monitored by routine prenatal checkups.

Statistical analyses

Statistical analysis was carried out by using a non-parametric Kruskal–Wallis test to evaluate differences of time from diagnosis of POI to ovariectomy and Fisher's exact test to evaluate differences of the frequencies of AMH concentrations above a threshold (0 pg/ml) between the groups. Fisher's exact test was used to compare the proportion of patients with endometriosis. Results for age at ovariectomy are presented as mean ± standard deviation, whereas data for time from diagnosis of POI to ovariectomy are expressed as median (quartiles).

Results

Vitrification of ovarian tissues from POI patients

The numbers of ovarian strips prepared from POI patients were limited when compared with those from 'normal' ovaries of cancer patients for fertility preservation (POI: mean 7.2 ± 5.4 strips, range: 1.5–25 per ovary, $n = 37$; cancer patient: mean 19.2 ± 5.1 strips, range: 11–30 per ovary, $n = 18$).

Auto-transplantation of ovarian cubes and monitoring of follicle growth

In patients with follicle growth in grafts beneath Fallopian tubes, growing follicles in the graft (Fig. 2B, left panel, plus signs) were detected based on their unique morphology characterized by the absence of a neighboring medulla. This is in direct contrast to the routine detection of growing follicles inside the ovary of infertile patients undergoing oocyte retrieval for IVF following controlled ovarian stimulation. For these follicles inside the ovary (Fig. 2B, right panel), adjacent medullar structure (arrows) and the outline of ovary (arrowheads) could be detected.

Histological assessment of residual follicles in ovarian cortices for vitrification

In our previous study (Kawamura *et al.*, 2013), follicle growth after IVA in POI patients was only detected in patients with residual follicles based on histological analyses of ovarian cortices. After enrolling 10 more patients, we re-evaluated if this approach could be a reliable parameter to predict the IVA outcome. In general, we used 10–20% of each ovarian strip to evaluate residual follicles. Although total numbers of residual follicles were different among patients, we could find a few primordial, primary and secondary follicles in sections from some patients (Fig. 3A, left upper and lower panels). In contrast, 46% of patients showed the absence of residual follicles even after extensive histological analyses

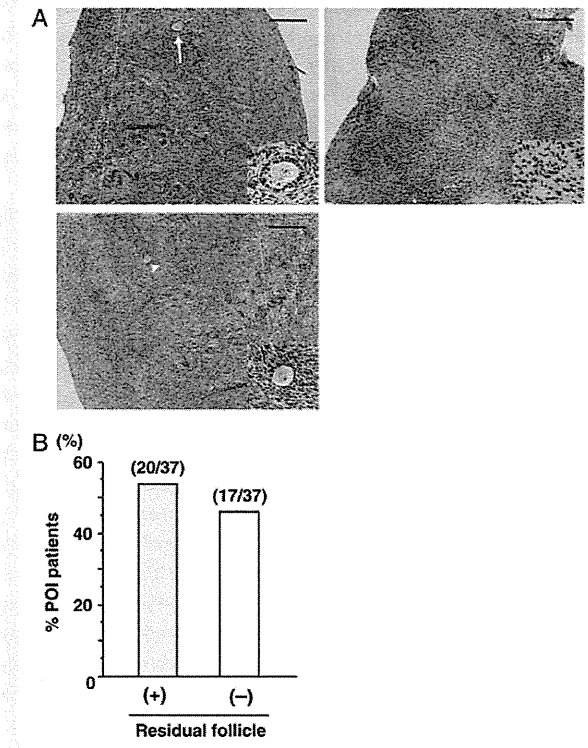

Figure 3 Histological analyses for the evaluation of residual follicles in ovarian cortical strips of POI patients. (**A**) Representative H&E staining images of ovarian cortical sections from patients with POI. Two left panels: a patient with residual follicles; Right panel; a patient without residual follicles. Arrow: secondary follicle; arrowhead: primordial follicle. Shown in inserts are high magnification images. Scale bars, 200 μm. (**B**) Proportion of POI patients with or without residual follicles. Numbers inside the parentheses represents number of patients in each group/total number of patients.

(Fig. 3A, right panel showing the presence of connective tissue cells alone in the ovarian cortex). Ratios of patients with or without residual follicles are shown in Fig. 3B. For 17 patients without residual follicles based on histological analyses, we performed auto-transplantation following patients' requests. However, none of these patients showed follicle growth during ~1 year of observation after transplantation. Thus, it is becoming clear that our histological analyses appear to be a valid parameter to predict potential IVA success.

Successful follicles growth after IVA followed by auto-transplantation

By adding 10 new patients to the cohort of 27 patients in a previous paper (Kawamura *et al.*, 2013), we now describe findings on 37 POI patients. Among them, ovaries from 20 out of 37 contained residual follicles based on the histological analyses. In patients with follicles, 9 out of 20 showed follicle growth in auto-grafts with 24 oocytes retrieved from six patients. Following IVF and embryo transfer into four patients, pregnancies were detected in three patients based on serum hCG, followed by one miscarriage and two successful deliveries. In addition to the first

healthy male baby born after IVA treatment (Kawamura *et al.*, 2013), a female baby (birthweight, 2970 g; and Apgar score, 8 at 1 min/9 at 5 min; blood pH level of umbilical artery, 7.27) was delivered at 38 weeks and 2 days of pregnancy by caesarian section. Physical features of the baby are normal, together with normal placenta (443 g) and umbilical cord. Checking the transplantation sites of ovarian fragments indicated no abnormal growth in the transplanted site beneath the Fallopian tubes.

Based on the clinical data and patient history, we attempted to identify additional parameters to predict IVA success. As shown in Table I, patient age at ovariectomy for vitrification was similar in all groups, but the duration from the diagnosis of POI to ovariectomy was significantly shorter in those patients with residual follicles that responded to IVA treatment. Among 37 POI patients, only nine patients showed detectable levels of serum AMH and patients with positive IVA outcomes had the highest median serum AMH levels (Table I). Among patients containing residual follicles, those responded to IVA treatment by showing follicle growth have median of serum AMH levels almost 2.3 times higher than those not responding to IVA treatment (Table I). However, there was no difference of the frequencies of AMH concentrations above a threshold (0 pg/ml) between groups with histological presence and absence of residual follicles (20 versus 29.4%), thus suggesting serum AMH levels are a secondary parameter for predicting IVA success when compared with screening using histological analyses. Interestingly, we frequently detected endometriosis in patients with residual follicles and responded to IVA treatment by showing follicle growth (Table I).

Discussion

We successfully cryopreserved functional early ovarian follicles using the vitrification protocol described above based on successful follicle growth after IVA and auto-grafting. Enrolling 10 more patients in addition to those described in our previous publication (Kawamura *et al.*, 2013), we demonstrated that histological analyses of ovarian cortices and the patients' history of duration from POI diagnosis to ovariectomy are associated with the IVA outcome.

Although we have not compared vitrification versus slow freezing for ovarian tissue cryopreservation here, our findings suggest that the present vitrification protocol is effective for ovarian tissue preservation. Cryopreservation of ovarian tissues is becoming an important option for fertility preservation in cancer patients. Its advantages include its availability to prepubertal girls and the storage of a large number of immature oocytes; moreover, cryopreservation of ovarian tissue minimizes the delay needed for freezing of mature oocytes or embryos. Ovarian tissue vitrification can be scheduled immediately, whereas substantial times are required for ovarian stimulation to obtain mature oocytes and embryos (Loren *et al.*, 2013). For cancer patients, ovarian tissue cryopreservation allows chemo- or radiation therapies to be started without delay. In the cases of POI, ovarian reserve, namely the pool of residual follicles, continues to diminish with age (Nelson, 2009). This is consistent with our findings showing that a shorter duration of amenorrhea from POI diagnosis to ovariectomy is associated with better responses to the IVA treatment. If one ovary is cryopreserved at an earlier age, patients could undergo additional non-invasive infertility treatments before the final decision for the IVA treatment. In addition, cryopreservation of ovarian tissues allows sufficient time for histological analyses to evaluate the presence or absence of residual follicles before a decision is made for auto-transplantation. It also provides the option to schedule the second surgery more conveniently.

The present approach represents a successful cryopreservation of ovarian tissues using vitrification for the generation of functional mature oocytes for infertility treatment. Based on our experience, it is important to avoid failure of vitrification during the step of cryoprotectant equilibration. This failure can be minimized by preparing thin layers of ovarian strips (Amorim *et al.*, 2011). For the vitrification of POI ovaries, special care should also be taken for tissue preparation due to the frequent presence of uneven ovarian surfaces caused by ovarian shrinkage, especially in advanced POI cases.

For transplantation of cryopreserved ovarian tissues, one could select the original site (orthotopic) for grafting, including the peritoneum of the ovarian fossa and the remaining ovary. Orthotopic site has the advantages of using the original niche for follicular development and the possibility for natural conception. At present time, more than a dozen of live births have been reported after auto-transplantation of cryopreserved ovarian tissues at orthotopic sites (Donnez *et al.*, 2004). For heterotopic grafting, the subcutaneous site is frequently chosen due to the involvement of minimal invasive surgical intervention and easy access for oocyte retrieval (Demeestere *et al.*, 2009). However, heterotopic grafting was less effective for successful pregnancy. Recently, one live birth was reported after grafting cryopreserved ovarian tissues at pockets on the rear side of the broad ligament, following promotion of neoangiogenesis by adding platelet-rich plasma containing high concentration of

Table I Possible parameters to evaluate successful follicle growth after IVA treatment.

Residual follicles at histology and growth after transplantation (*n*)	Age at ovariectomy (*y*)	Time (*y*) from diagnosis of POI to ovariectomy	Number of patients with AMH before ovariectomy (pg/ml)*	% patients with endometriosis (*n*)
None (17)	37 ± 4.7 (28–43)	7.5 (2.5–12.1)[a]	5 (196)	6 (1)[b]
Present but no growth (11)	37 ± 4.9 (31–48)	5.0 (3.0–5.5)	1 (170)	0 (0)
Present and growth (9)	39 ± 4.4 (29–42)	1.0 (0.5–4.7)[a]	3 (390)	44 (9)[b]

Data for age at ovariectomy are mean ± SD (range), whereas data for time from diagnosis of POI to ovariectomy are median (quartiles). For anti-Mullerian hormone (AMH), the number of patients with detectable AMH and median of serum AMH levels among these are shown.
*Data (pM) obtained by Active MIA/AMH EIA were converted into the unit (ng/ml) used for the AMH Gen II ELISA by a multiplication factor of 0.14 but are shown as pg/ml for clarity. Statistical analysis was carried out by using a non-parametric Kruskal–Wallis test to evaluate differences of time from diagnosis of POI to ovariectomy and Fisher's exact test to evaluate differences of the frequencies of AMH concentrations above a threshold (0 pg/ml) between the groups. Fisher's exact test was used to compare the proportion of patients with endometriosis. [a,b]Values in the same column with different superscripts are significantly different ($P < 0.05$).

pro-angiogenic growth factors (Callejo *et al.*, 2013). We chose the serosa of Fallopian tubes as the auto-grating site due to its high vascularity and the ease to monitor follicle growth. Because we observed minimal bleeding after surgical cutting of POI ovaries during removal, we hypothesized that the remaining ovary is not optimal for the revascularization of transplanted tissues. Future studies are needed to evaluate the best autografting sites for ovarian tissues.

As indicated earlier (Kawamura *et al.*, 2013), we started with bilateral ovariectomy for IVA treatment but subsequently recommended the removal of one ovary because only small numbers of vitrified ovarian strips are needed for the IVA procedure and many patients expressed the intention to retain one ovary. Following cutting ovarian strips into cubes (Hippos signaling disruption) and Akt stimulation using IVA drugs, multiple antral follicles were detected in 45% of POI patients containing residual follicles. Twenty-four oocytes were retrieved from six patients and 15 embryos were derived from six patients. Among them, embryo transfer was performed for four patients and ongoing efforts are being made to generate more oocytes and embryos for the remaining patients. Three patients became pregnant based on elevated serum hCG levels. Among these three patients, one had a miscarriage and two gave birth. Although two healthy babies were born and the first baby showed normal development during 22 months after birth, more follow-up studies are required to ensure the safety of the present IVA procedure.

It is well-known that the quality of oocytes decreases with age (Broekmans *et al.*, 2009) and it is important to note that the present IVA treatment mainly promotes follicle growth and is unlikely to correct any age-related decline in egg quality. For POI patients, who are candidates for IVA treatments, cryopreservation of ovarian tissues at a younger age is highly recommended for the minimization of age-related increases in genetic defects in oocytes.

The semi-quantitative histological analyses show the strongest association with IVA outcome. For non-invasive approaches to predict the presence of residual follicles, our data suggested that high levels of serum AMH are associated with a better IVA outcome. However, some patients with undetectable AMH levels could still respond to the IVA treatment. In the human ovary, AMH expression was negligible in primordial follicles, low in granulosa cells of primary follicles but highest in granulosa cells of secondary, pre-antral and small antral follicles $\leq$4 mm in diameter. In larger antral follicles, AMH expression gradually disappeared (Weenen *et al.*, 2004). Because the IVA treatment presumably promotes the development of primordial, primary and secondary follicles, patients with residual primordial and primary follicles could also respond the IVA treatment. Thus, our findings indicated that serum AMH is not the best predictor for treatment outcomes.

A shorter duration from diagnosis of POI to ovariectomy is was also associated with the likelihood of follicle growth. For the treatment of symptoms of estrogen deficiency, POI patients often received cyclic estrogen and progesterone replacement, leading to the induction of artificial menses. As a result, it is sometimes difficulty to know the exact duration of amenorrhea after POI diagnosis. Thus, in this study, we used the duration from the diagnosis of POI to ovariectomy as one of the parameters.

We also found prevalent endometriosis in patients responding to IVA treatment. Vascular endothelial growth factor (VEGF) is the most prominent pro-angiogenic factor shown to promote angiogenesis in endometriosis (Taylor *et al.*, 2002). Of interest, promotion of neovascularization at ovarian transplantation sites by creating peritoneal wounds before grafting (Donnez *et al.*, 2004) and by using platelet-rich plasma containing pro-angiogenic growth factors (Callejo *et al.*, 2013) have been found to enhance ovarian grafting success. One can speculate that VEGF derived from endometriosis might contribute to a better outcome of IVA. However, the number of patients under investigation here is too low and future expansion of IVA treatments is needed to evaluate if inclusion of angiogenic factors is useful. Also, future studies are needed to identify additional markers to indicate the presence of residual follicles in POI and to predict IVA treatment outcome using non-invasive approaches. Different oocyte-derived factors, including GDF-9, BMP15 and R-spondin2 (McGrath *et al.*, 1995) are potential candidates due to the larger size of oocytes when compared with small numbers of surrounding somatic cells in pre-antral follicles. Development of ultrasensitive assays are needed to detect the presumably low 'leakage' of these secreted factors into the systemic circulation.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

K.K. designed the study and wrote the manuscript. N.S., N.Y., S.T., Y.S., M.T., S. H., Y.M. and K.K. conducted the studies. N.S. participated in critical discussions and manuscript editing.

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Conflict of interest

None of the authors has a conflict of interest.

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human reproduction INVITED COMMENTARY

Ovarian tissue cryopreservation using vitrification and/or *in vitro* activated technology

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Slow freezing has been a standard method for ovarian tissue cryopreservation and transplantation (OTCP), while vitrification is commonly used for cryopreservation of embryos, oocytes and sperm. We have conducted preclinical studies using cynomolgus monkeys since 2006 (Hashimoto *et al.*, 2010; Suzuki *et al.*, 2012) and clinical studies since 2010, reporting a live birth after OTCP with vitrification (Kawamura *et al.*, 2013; Suzuki *et al.*, 2015). As discussed in their opinion article in this issue of *Human Reproduction* (Meirow *et al.*, 2015), it seems to be difficult to compare slow freezing with vitrification and determine which is better. The live birth rate might be thought of as a useful parameter for comparing the two procedures, but it is not actually very useful because the number of primordial follicles in ovarian tissues cannot be counted before transplantation. Vitrification can be performed quickly and easily using a commercially available kit without the need for expensive equipment. Such convenience is a great advantage. However, it is important to determine the optimal conditions for vitrification by taking into consideration the safety and efficacy of cryoprotectants.

It may be considered preferable to avoid OTCP in patients with premature ovarian insufficiency (POI) because they only have a small number of primordial follicles. However, we have performed '*in vitro* activated' (IVA) OTCP (Kawamura *et al.*, 2013; Suzuki *et al.*, 2015) in patients with POI for two reasons. First, POI is a progressive condition and the number of primordial follicles continues to decline. If an ovary is cryopreserved, hormone therapy can be given to induce follicular development and preparations can be made for IVA. Second, ovarian tissues need to be cultured for 48 h for IVA. Because all of the ovarian tissues harvested cannot be activated at the same time, some tissue must be cryopreserved without culture. It is preferable to perform IVA OTCP after harvesting, but residual ovarian tissue needs to be cryopreserved even in such a case. Meirow *et al.* are concerned about the safety of performing IVA OTCP after one to two cycles of chemotherapy. We do not think that IVA OTCP should be performed near the start of chemotherapy and have never performed it after one or two cycles of treatment. It is recommended that IVA OTCP should be performed when the patient is not undergoing chemotherapy because of the risk of birth abnormalities as Meirow *et al.* stated. We think that IVA OTCP should be performed in patients with progressive POI, including those with mosaic Turner syndrome, and in cancer patients aged 35 years or older. The International Society for Fertility Preservation (ISFP) and other reports recommend that OTCP should be performed at up to the age of 35–37 years because the number of primordial follicles is reduced by nearly 90% around that age (von Wolff *et al.*, 2011; ISFP Practice Committee, 2012; Wallace *et al.*, 2014). The number of primordial follicles is the key determinant of whether live birth can be achieved after OTCP and the possibility of successful live birth after OTCP increases if patients are younger. When patients are in their late 30s the live birth rate is low after freezing unfertilized eggs, while if fertilized eggs are frozen a considerable number of eggs have to be frozen. Thus we consider that cancer patients aged 35 years or older should try to give birth using IVA OTCP as soon as possible after achieving remission with cancer treatment, and thereafter should undergo conventional OTCP to extend graft survival and obtain normal endocrine function for a long time regardless of their age or the type of cancer. However, there is concern that PI3K/PTEN signaling may induce tumor cell growth in ovarian tissue during IVA. If OTCP can be done in cancer patients without minimal residual disease, then IVA OTCP can be performed under treatment with a PI3K stimulator and PTEN inhibitor. It may be possible to only perform ovarian fragmentation to disrupt Hippo signaling in patients who should have children and raise them as quickly as possible. We are currently investigating the usefulness of transplanting cryopreserved ovarian tissue in cancer patients by using two OTCP methods. Donnez *et al.* have brought a splendid breakthrough in this field, which is beneficial for young cancer patients around the world (Donnez *et al.*, 2004). We hope that the addition of vitrification and/or IVA for OTCP will enable more cancer patients to have children in the future.

Author's role

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がん・生殖医療連携体制の国内外の動向

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『がん・生殖医療』とは，“妊孕性温存療法”のみではなく，がん患者がそれぞれの将来の妊娠・出産について最良の選択をするために支援してゆく治療全般を指す。そのため，その実践には各診療科の医師だけでなく，看護師や心理士などの様々な専門性をもった医療者の参画と，連携体制の構築が不可欠である。また，地域における施設間のネットワークも非常に重要であり，わが国でも徐々に誕生しつつある。さらに日本がん・生殖医療研究会では，医療者間の連携だけではなく，患者ネットワークや厚労省研究班との連携を構築することによって，『がん・生殖医療』をより有効なものにできるような取り組みを行っている。様々な連携体制が構築された先に，より成熟した『がん・生殖医療』の姿があると考えられる。

はじめに

わが国において『がん・生殖医療』という言葉が生まれてから，はや5年が経とうとしている。本稿では，これから成熟期を迎えようとする，『がん・生殖医療』のわが国における現状を見つめ直し，その連携構築にむけた諸問題について概説する。

1．『がん・生殖医療』とは

『がん・生殖医療』という言葉は，2006年に，アメリカの科学者であるWoodruffらが提唱したOncofertilityという言葉が由来となっている。Oncofertilityは，腫瘍学(Oncology)と生殖医療(Fertility treatment)が組み合わさってできた造語であり，独立した専門分野である両者を結びつけることによって，がん治療の世界では顧みられることが少なかった，がんサバイバー(Cancer survivor)における『妊孕性喪失』という問題に対して目を向けさせた。その結果，若年がん患者が妊孕性を喪失する可能性のある化学療法や放射線療法を施行される前に，Fertility preservation，つまり妊孕性温存療法(一般的には卵子や受精卵・精子の凍結)が行われるようになった。これらは，特に生殖医療の技術革新に負うところが大きく，それ無しには今日のような妊孕性温存療法の発展は成しえなかったといえる。さらに近年では，1998年に凍結卵巣組織からの出産例がDonnezらによって初めて報告されて以来[1]，すでに約40名の出生例が報告されており，わが国においても出産例が報告されている[2]。また，男性における妊孕性温存療法は精子凍結がメインであったが，今日では精巣凍結による産仔獲得例がマウスにて報告され[3]，実験的でありながらも小児悪性腫瘍患者に対して臨床応用されつつある。

しかしながら，筆者らが考える『がん・生殖医療』とは，もっと広範なものを指す。例えば，婦人科領域に特有の女性性器悪性腫瘍に対する妊孕性温存手術が挙げられる。古くから施行さ

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れている円錐切除術や子宮筋腫核出術，卵巣腫瘍摘出術は元来機能温存手術であり，広義の意味での妊孕性温存手術であった。さらに近年では適応の限界はあるものの，広汎性子宮頸部摘出術(trachelectomy)が普及しつつあり，広汎性子宮全摘術と遜色ない治療効果が認められている[4]。つまり，生殖医療だけでなく，手術療法を含めたがん治療のなかにも『がん・生殖医療』が存在する。まだ実験的ではあるものの，放射線科領域においても，全身放射線療法時に『卵巣遮蔽』を行うことで卵巣へのダメージを減らす試みがなされており，その成功例に関する報告もなされている[5]。さらに悪性腫瘍に対する化学療法においても，治療効果は同等であるが，アルキル化剤などの卵巣毒性の強い薬剤を使用しないレジメンの開発が取り組まれており，このような取り組みも『がん・生殖医療』であると考えられる。

また，『がん・生殖医療』として忘れてはならないものに，Cancer survivorを対象とした生殖医療が挙げられる。Cancer survivorに対する生殖医療の特殊な点として，乳がんのようにエストロゲンレセプターをもつ組織型の場合には，生殖医療を行うことで腫瘍が再発する可能性がある。また白血病のように，卵巣に悪性腫瘍細胞，つまり微小残存癌病巣(Minimal Residual Disease；MRD)が高率に存在することがわかっている腫瘍では[6]，卵子凍結や受精卵凍結を行う際の採卵によって腫瘍細胞が転移する可能性がある。なお血液疾患全般に言えることではあるが，採卵時のヘモグロビン値・白血球数・血小板数によっては，採卵などの侵襲的な治療ができなくなることもある。このように，特殊な状況下での生殖医療も『がん・生殖医療』の一面であると考えられる。さらに，がん治療によって卵巣機能が著しく弱まってしまった，つまり原始卵胞が極端に減少した結果，早発卵巣不全(primary ovarian insufficiency；POI)もしくは早発閉経(Premature ovarian failure)をきたしてしまった患者に対する生殖医療も，『がん・生殖医療』の一部であると考えられる。

そのほかの『がん・生殖医療』の重要な要素として，看護ならびに心理的な支援が重要と考えられる。生殖外来を受診したがん患者は一般的に，治療開始前に本当に妊孕性温存療法を行うか否か，どのオプションを選択するのか，といった様々なことを短時間で決めなければならない。ただでさえ，がんに罹患したという極めてストレスの大きな精神状態のなか，卵巣へのダメージと将来の不妊の可能性についてきちんと理解して最良の選択をすることは，非常に困難なことである。医師からの説明だけでは不十分な場合もあり，患者も特に男性医師には聞きづらい事柄もあるだろう。それを補完するのが，看護師であり心理士やケアマネージャーである。米国ではこのような役割を担う医療者を“Health care provider”と称し，“Oncofertility”において極めて重要な役割を担っている。

以上のように『がん・生殖医療』とは，ただ単純に妊孕性温存療法を提供するだけの医療ではなく，様々な職種の医療者がそれぞれの特性を活かし，がん患者が最良の選択をできるように支援してゆくものである。このように，『がん・生殖医療』はまさしく“医療”であり，その実践には医師だけでなく，様々な医療者の協力が不可欠と考えられる。

2．わが国における『がん・生殖医療』の現状

わが国において『がん・生殖医療』という言葉が生まれたのは約5年前であるが，それまでは個々の施設において妊孕性温存療法が行われてきた。そのような取り組みが初めて社会に出てきたのが，2007年にA-PART日本支部(現日本A-PART)が開始した，『複数施設における悪性腫瘍未婚女性患者における卵子採取，ならびに凍結保存の臨床研究』(研究代表者：宇津宮隆史先生)であると思われる。A-PART日本支部は，民間不妊治療施設の交流によって結成された団体であり，卵子凍結がまだ“実験的な医療”であった2007年にこのような先進的な臨床試験が民間施設において開始されたことは特筆