選択することが不可欠である。現在，胚のクオリティー評価は簡単で非侵襲的な方法である形態評価法が最も普及している。しかし，形態評価法は観察者の主観により判定結果に差が生じる可能性があるため，より客観的で，精度が高く，安全な胚クオリティー評価法の開発が望まれている。

筆者らは形態観察に比べてより客観的な方法として，細胞呼吸能測定による胚クオリティー評価方法を提案している。これは，高感度で生体反応を検出できる電気化学的計測技術の基盤である走査型電気化学顕微鏡（SECM）を用いて非侵襲的に胚の呼吸量を測定し，呼吸能を指標に胚のクオリティーを評価する新しい方法である。これまでにウシ胚を用いた研究により，呼吸活性を基準とする胚クオリティー評価法の開発に成功している[1, 2]。さらに，SECMを用いて単一のヒト胚の呼吸量測定に初めて成功した[3]。そこで本研究は，SECMを用いた胚の評価が安全でかつ着床能を有する胚の選択が可能となるかを検討するために，まず胚の発育を観察した。Day 3胚（体外受精：Day 0）の呼吸量を測定後，追加培養を行い，呼吸量測定胚と非測定胚の発育能を比較することで，胚への影響を調査した。胚呼吸量の違いと胚発育の関係についても検討した。また，物理的影響を避けるため，最適顕微鏡ステージ温度についても検討を行った。

方　法

患者背景および測定方法

体外受精胚移植または凍結胚移植を施行し，患者の同意が得られた未移植胚を研究に用いた。対象期間は2006年7月から2007年7月で73周期188個の胚を測定した。平均年齢は34.5 ± 4.5歳で平均体外受精施行回数は2.7 ± 2.3回だった。受精後1日から3日までは，Sydney IVF Cleavage Medium（Cook社）を用いて5% O_2, 6% CO_2, 90% N_2の気相下に37℃で培養を行った。受精日をDay 0とし，Day 3にて4～10細胞期胚に発生した胚をVeeckの分類法[4]を基に評価した。胚を形態観察により評価した後，SECMをベースに開発した「受精卵呼吸測定装置」を用いて個々の胚の呼吸量を測定した。呼吸測定後，胚発生能を調べるために個々の胚をSydney IVF Blastocyst medium（Cook社）に培養液の交換を行い，Day 5–7まで培養した。装置は，倒立型顕微鏡，ポテンショスタット，呼吸解析ソフトを内蔵したノートパソコンにより構成されている。呼吸量の測定には，マイクロ電極と参照電極，測定プレートを用いた。測定プレートに10% Synthetic serum substitude（SSS；IS Japan）を添加したmodified-Human Follicle Fluid（m-HFF；扶桑薬品工業株式会社）を5 ml入れ，マイクロウェルの中にミネラルオイルが混入しないように胚を洗浄した後，マイクロウェルの底部中心に胚を静置した。参照電極を測定プレートの端に設置した後，マイクロ電極を透明帯の間近に移動させた。ポテンショスタットの電位を－0.6 V vs. Ag/AgCl（参照電極）に保持した後，移動速度30 μm/sec，走査距離160 μmの条件で透明帯に対して鉛直方向（Z軸方向）に掃引した（図1）。1個の胚に対する測定時間は約30秒である。測定中のマイクロウェル内温度や浸透圧変化を最小に留めるため，最適顕微鏡ステージ温度の検討を行った。浸透圧測定にはOSMOSTAT（アークレイ株式会社）を用い測定を行った。

結　果

顕微鏡ステージ温度について検討を行った。経時的に温度と浸透圧変化を調べた（図2）。40度に顕微鏡ステージ温度を設定した場合20分以後より温度低下を示し，45度では5分後より温度の上昇を認めた。浸透圧変化では，45度に設定した場合15分後には290 mOsm/kgを上回り上昇が早くなる結果を示した。42.5度では培養液の液温も35度を保ち浸透圧も30分までは300 mOsm/kgを上回ることはなく，比較的最適な条件を長く保つことが可能であると考えられる。

呼吸量測定による，胚発育への影響を評価するために呼吸量測定群と呼吸量非測定群で胚盤胞発生率も比較した。呼吸量測定群で胚盤胞発生率は57.0%，非測定群で51.8%と差は認めなかった（図3）。

同一分割期において，それぞれの胚の呼吸能に顕著な違いが認められた。例えば，Veeckの分類によって4分割グレード1と形態的に同じクオリティーと評価された胚の呼吸量を比較した結果，呼吸測定値はそれぞれ①0.25，②0.44，6分割グレード2では③0.57，④0.23，8分割グレード2では⑤0.71，⑥0.35であり胚によって顕著な違いが認められた（図4）。このように本研究では，Veeckの分類法による形態的評価と胚の呼吸能には明確な相関は認められなかった。Day 3における4～10細胞期胚（n=187）の呼吸量を測定した。各分割期の酸素消費（呼吸）量を表1に示す。呼吸量（$F \times 10^{14}$/mol s^{-1}）の平均値は，0.34–0.50であり各分割期間に顕著な差はなかった。Day 3胚の呼吸量測定後，胚盤胞期まで培養し，呼吸能と胚盤胞発生率の関係を調べた。その結果，Day 3において呼吸量が0.26-0.56 $\times 10^{14}$/mol s^{-1}であった胚は65.8%が胚盤胞に発生した。一方，呼吸量が0.26 $\times 10^{14}$/mol s^{-1}未満及び0.56 $\times 10^{14}$/mol s^{-1}より大きい場合の胚盤胞発生率は，39.0%であった（図5）。

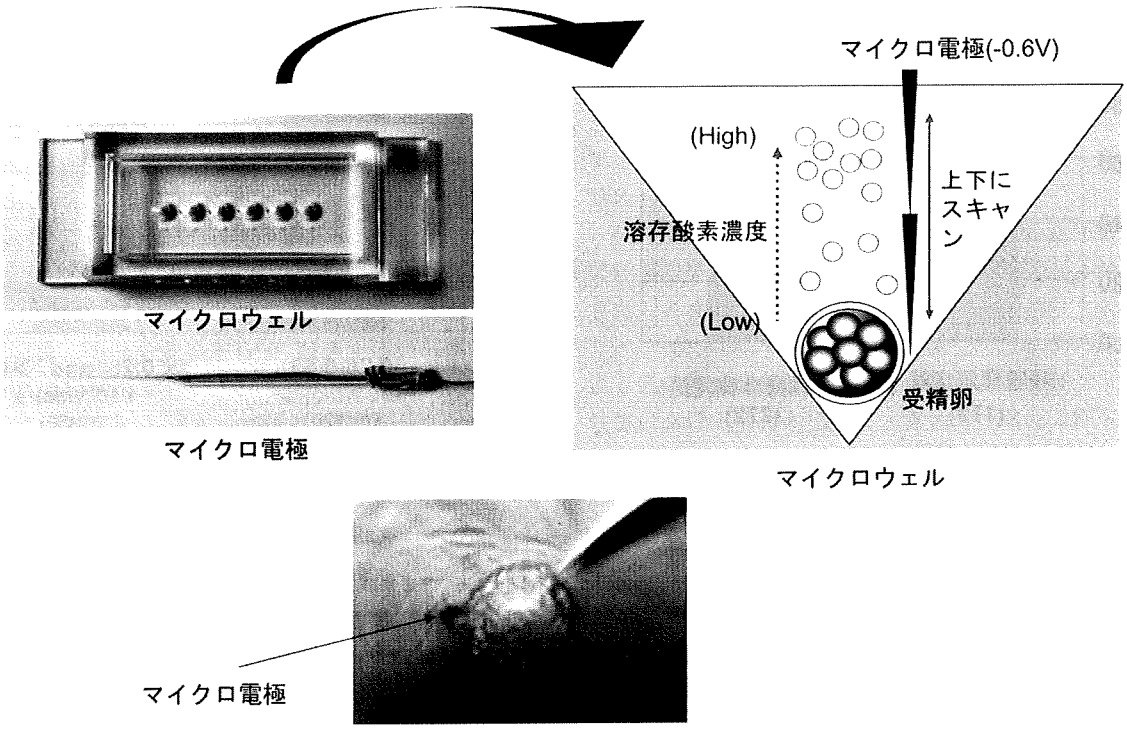

図1　SECMを用いた呼吸量測定

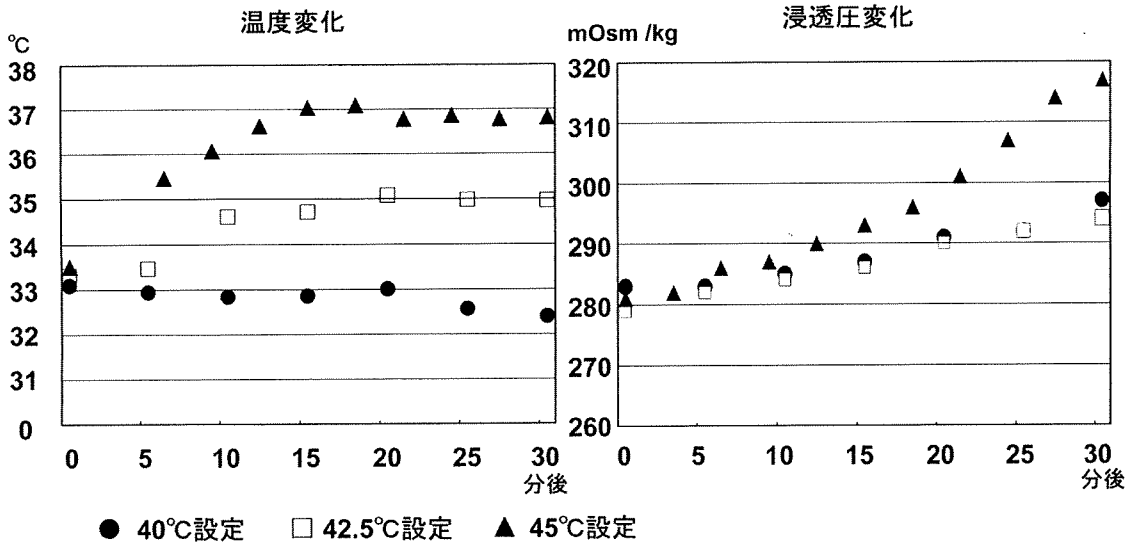

図2　顕微鏡ステージ温度における培養液変化

考　察

胚移植にあたり，最も良好な胚を選別し，1個の移植をすることは多胎率の低下のために不可欠である。これまで，分割期胚の段階でクオリティー良好胚を選別するための形態的判断基準として多くの施設でVeeckの分類[4]が使用されている。さらに，よりクオリティー良好な胚を選択するために様々な判定基準について研究が進められてきた。Zollnerら[5]は前核の接着や前核数の評価に基づく，スコアリングシステムを開発・提案している。

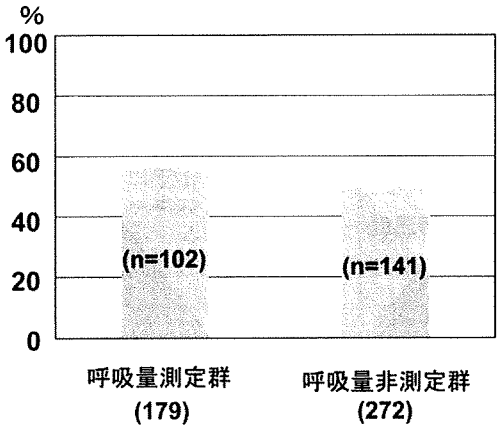

図3　呼吸量測定，非測定群における胚盤胞発生率

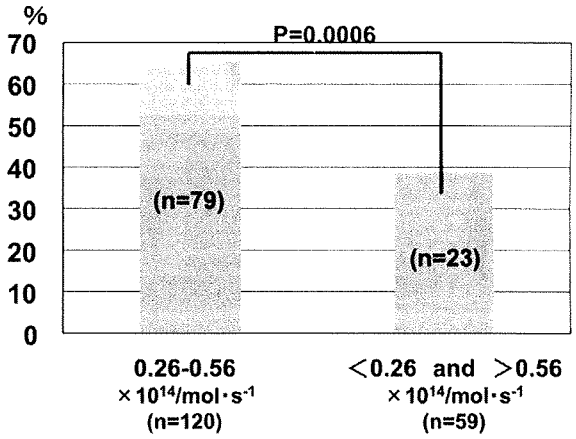

図5　ヒト胚の呼吸能と胚盤胞発生率の関係

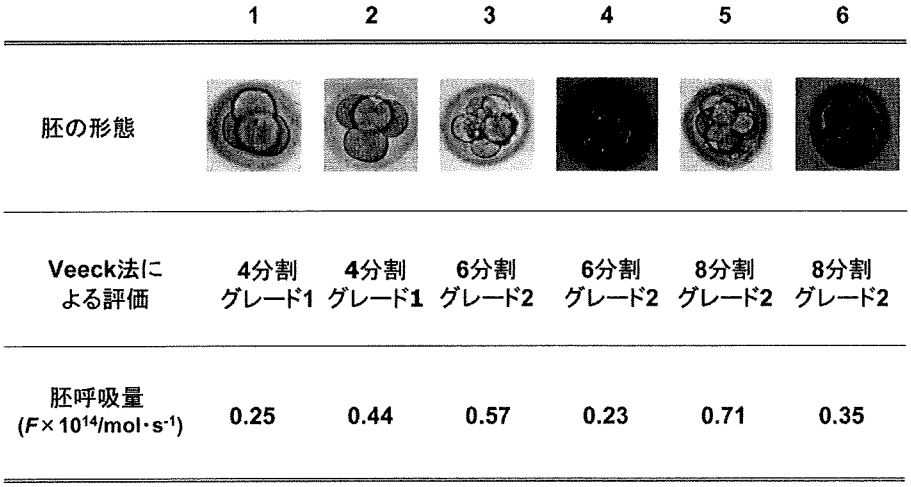

図4　ヒト体外受精胚の形態と呼吸量

表1　異なる分割期におけるヒト胚の酸素消費量（呼吸量）

分割期	測定胚数	呼吸量 ($F \times 10^{14}$/mol·s^{-1})
4細胞	8	0.34 ± 0.1
5細胞	15	0.45 ± 0.2
6細胞	39	0.37 ± 0.1
7細胞	51	0.39 ± 0.2
8細胞	50	0.40 ± 0.2
9細胞	12	0.40 ± 0.1
10細胞	12	0.50 ± 0.2

N.S

Scottら[6]，Tesarik and Greco[7]や公文ら[8]は，前核や核小体の形態による判定を提案している。また，Cirayら[9]は，2細胞期のそれぞれの細胞の核の局在の判定と早期分割による胚発生能の評価法を提案している。しかし，いずれも形態的特徴の観察による評価法であるため，判定結果が観察者の主観によって影響を受ける可能性は否定できない。1998年頃からは，培養期間が長くなることにより移植胚の選別が容易になると考えられ，胚盤胞期移植が広まった。しかし，一卵性双胎が増加することや，培養期間が長くなるため，胚移植キャンセルになる可能性が高くなるという欠点も持ち合わせている。そのため，大多数の施設ではDay 2もしくはDay 3の分割期胚を移植しているのが現状である。分割期胚移植と胚盤胞移植の比較検討としては，Gardnerら[10]やScholtesら[11]が，胚

盤胞移植を行うことにより着床率は分割期胚移植に比べて有意に上昇したと報告をしている。しかし，Coskunら[12]，Karakiら[13]やUtsunomiyaら[14]により，胚盤胞まで発育が進まず移植キャンセルとなった症例を含めた上での妊娠率は分割期胚移植と有意差はないという報告もなされている。分割期胚移植の検討としては，受精確認からDay 2移植もしくはDay 3移植の妊娠率の比較を行った結果，Carilloら[15]の妊娠率に差があるという報告の一方，Lavergeら[16]は妊娠率に差はないという報告をした。

阿部らは，胚のクオリティーに関連する微細構造の解析を行い，ミトコンドリアが胚のクオリティーに密接に関係していることを発見している[17]。ミトコンドリアは酸化的リン酸化反応（呼吸）により細胞活動に必要なエネルギー（ATP）を産生することから，ミトコンドリアが発達している胚は呼吸活性が高く，一方の不良胚ではミトコンドリアの呼吸代謝能が低いと考えられる。この研究成果を基に阿部らは，高感度・非侵襲的に細胞の呼吸を検出できる電気化学計測技術に着目し，この計測技術の中心であるSECMをベースとする受精卵呼吸測定装置を開発した[1, 18, 19]。これまでに，ウシ，ブタ，マウスの胚の無侵襲的呼吸能解析に成功しており，呼吸能を指標とする胚クオリティー評価法の有効性を示している[2]。最近，電気化学的呼吸計測技術のヒト胚への応用を目的に，ヒト余剰胚の呼吸量測定を行っている。体外受精後の全ての発生ステージにおいて胚の呼吸量測定に成功するとともに，ミトコンドリアの発達と呼吸量の増加が一致することを明らかにした[3]。また，以前に我々はヒト胚の形態と呼吸能との関連性を調べた結果，胚の呼吸能とVeeckの分類法による形態的評価は必ずしも一致することはないことを示し，電気化学計測法は，形態観察では評価できない胚の呼吸能をモニタする可能性を報告した[20]。

今回我々はこの呼吸計測技術が胚へ及ぼす影響を調査するとともに胚評価の可能性についても検討を行った。呼吸量計測に用いるマイクロ電極はピコアンペアレベルの電流の変化を検出できる。この微小電極の周りに生じる電場は電圧が0.1 μV以下電流は1 nA以下であり，細胞の膜電位（60–90 mv）の60万分の1以下のため胚への影響は無いと思われる。測定時の温度や浸透圧変化を考え測定プレートを保温する顕微鏡ステージの最適温度についても検討した。40度，42.5度，45度で検討を行ったが，40度では測定プレート内温度の低下が見られ，45度では温度に問題はないものの，浸透圧の上昇が早くなった。m-HFFの指示する浸透圧は275–295 mOsm/kgである。そこで我々は42.5度に設定し呼吸量測定を行った。1個の胚に対し測定に要する時間は30秒だが，マイクロ電極を胚の透明帯近傍に静置すること，次の胚へ電極を移動する動作も含めると1個の胚への所要時間は1分以内である。また，1回の測定で測る胚の個数も少数に制限している。呼吸測定を行った胚への影響は胚盤胞の発生率を以って評価したが，呼吸量測定群，非測定群で差は認められなかった。

胚呼吸量と胚発育の関係では呼吸量0.26–0.56を示す胚で胚盤胞発生率がそれ以外と比較して高くなる結果を示した。これはミトコンドリアによる呼吸活性と胚のクオリティーが密接に関係している可能性がある。呼吸量が0.26より低い胚は胚盤胞への発育が低い。これはミトコンドリアにおける活性が低いことを示しており，胚発育へ影響を与えていることが考えられる。呼吸量が0.56より高い胚でも胚盤胞への発育が低い。阿部らはクオリティー良好胚では，桑実胚から胚盤胞期にかけて多くのミトコンドリアにおいてサイズの増加やクリステの拡張が認められ，呼吸量が増加していくことを報告している[21]。このことから，胚発育が低い原因としてDay 3時の早い時期にミトコンドリアが発達し過ぎていることも考えられ，胚盤胞へ到達する前に活性が終息してしまうかもしれない。その結果，呼吸活性が低すぎても高すぎても胚盤胞発育が低くなる結果を示したのではないかと思われる。

本研究では，電気化学的呼吸測定技術を応用した新しいヒト胚クオリティー評価の安全性と可能性が示された。胚の呼吸測定と形態的評価を併用することで，より厳密に胚のクオリティーを評価できる可能性がある。今後は妊娠率を含めた臨床的有用性を検討していく必要があると思われる。

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—Technology Report—

Viability of Porcine Embryos after Vitrification Using Water-soluble Pullulan Films

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Abstract. The efficiency of a porcine embryo vitrification method that uses water-soluble films of pullulan, a naturally-occurring polysaccharide polymer, was compared with two other types of vitrification methods using different devices and solutions for vitrification and warming. Blastocysts collected *in vivo* and vitrified by the conventional straw (ST), Cryotop® (MVC) or pullulan film vitrification (PFV) methods were stored in liquid nitrogen for a certain period of time, after which the cryoprotective agents were removed by stepwise dilution. Fresh embryos were used as controls for the non-vitrification group. The vitrified-warmed embryos were incubated in TCM199 with 0.1 mM β-mercaptoethanol and 20% fetal bovine serum for 24 h at 38.5 C in humidified air with 5% CO_2 to evaluate their viability. The survival rate of embryos in the ST group (48.3%) was significantly lower than that of those in the MVC (70.7%), PFV (79.0%) and non-vitrification (94.4%) groups. The oxygen consumption rate after vitrification was significantly lower than that before vitrification in the ST group, but was not significantly different in the MVC and PFV groups. Both the oxygen consumption rates of embryos after warming and the live cell numbers in the ST group were lower than those in the MVC group, while they did not differ significantly between the PFV and MVC groups. There was a correlation between the oxygen consumption rate and the number of live cells in vitrified embryos after warming. Our results demonstrated that *in vivo*-derived porcine embryos could be vitrified using pullulan films.

Key words: Oxygen consumption rate, Porcine embryo, Pullulan film, Vitrification

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Preservation of porcine embryos is important for increasing the effective use of high-quality genetic resources, preventing disease transmission via animals and allowing low-cost transportation of pigs. Due to the extremely high sensitivity of porcine embryos to low temperature, it has been difficult with slow freezing methods to achieve conception rates and litter sizes equivalent to those achieved with artificial insemination [1, 2]. However, it has recently become possible to produce piglets from cryopreserved embryos by using ultra-rapid vitrification methods, such as the minimum volume cooling (MVC) [3, 4], open pulled straw [5], microdroplet [6] and metal mesh vitrification [7] methods.

Pullulan, which is a neutral polysaccharide polymer also known as α-1,4-; α-1,6-glucan, is made from starch and consists of maltotriose units linked in an orderly manner. It has been reported that murine morulae placed on a pullulan film could be vitrified [8]. In general, stepwise dilution is required after warming of vitrified embryos, since vitrification requires a high concentration of cryoprotective agents (CPAs), which makes it difficult, due to their toxicity, to warm the embryos in a straw for direct transfer to recipients. As the pullulan film is soluble in warm water, the vitrification solution can be diluted in a straw, and then the embryos can be directly transferred into a recipient using a straw-attached intrauterine injector.

Several studies on non-surgical embryo transfer in pigs have been reported [9–13], and we have also been working on the development of a non-surgical transfer technique that uses a straw-attached intrauterine injector [14]. Direct transfer via a straw containing vitrified embryos with high viability may be applied to piglet production by non-surgical embryo transfer in an ordinary commercial farm.

Measuring the oxygen consumption rate using a scanning electrochemical microscope may be effective as a non-invasive evaluation of embryo quality [15]. We have previously reported that bovine embryos having a high oxygen consumption rate showed a high conception rate after embryo transfer [16]. In an attempt at embryo cryopreservation using the slow-freezing method, the oxygen consumption rate in embryos significantly decreased after freezing and thawing, indicating that the embryos were damaged by the cryopreservation procedure [16]. However, there are no reports available concerning the oxygen consumption rate before and after cryopreservation with *in vivo*-derived porcine embryos.

In the present study, to investigate the potential for application of the pullulan film vitrification (PFV) method in a non-surgical transfer technique for porcine embryos, we compared the survival rate of porcine embryos preserved by the PFV method with those attained by conventional vitrification methods. We also measured

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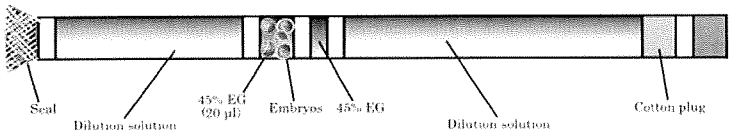

Fig. 1. A straw containing four liquid layers: (from the cotton-plug side) a dilution solution (PB1 + 1.7 M galactose + 0.5% BSA), a 45% EG vitrification solution (PB1 + 45% EG + 7% PVP + 0.5% BSA) that prevents reduction in the concentration of the vitrification solution due to mixing with the diluents of the first layers, a 45% EG vitrification solution containing embryos and a dilution solution. The straw was sealed after being filled with the last dilution layer.

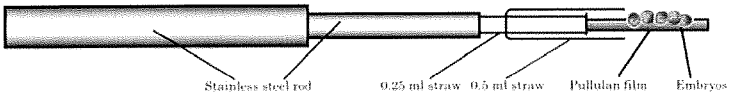

Fig. 2. A stainless steel rod for vitrification equipped with a pullulan film. A stainless steel rod was attached to a short 0.25-ml straw, and a pullulan film was attached to the tip of the straw. The 0.25-ml straw and pullulan film were covered with a case made from a 0.5-ml straw. Embryos and vitrification solution were placed on the pullulan film, and after the embryos were vitrified in liquid nitrogen, the case was slid across to cover the film.

the oxygen consumption rate of embryos before and after vitrification to examine how the rate was influenced by these vitrification methods.

Materials and Methods

Embryo collection from gilts

All animal-related procedures followed in this study were done with the approval of the Institutional Animal Experiment Committee of Kanagawa Prefectural Agriculture Facilities. A total of 39 prepubertal gilts (Landrace and Large White, 5.5–7.3 months old) were used for collection of embryos, as previously described with some modifications [13]. Briefly, superovulation was induced by intramuscular injection of 1500 IU eCG (Peamex, Sankyo, Tokyo, Japan), followed 72 h later by 500 IU hCG (Puberogen, Sankyo). The gilts were artificially inseminated twice, in the afternoon one day after hCG injection and in the morning two days after hCG injection.

In the morning on Day 6 (Day 0=the day of the first artificial insemination), the embryos were recovered from the uterine horns by laparotomy under general anesthesia (4–5% [v/v] halothane) by flushing with PB1 [17] with 0.5% (w/v) bovine serum albumin (BSA; Fraction V, Sigma Chemical, St. Louis, MO, USA) or TALP-Hepes [18]. The recovered embryos were morphologically evaluated under an inverted microscope (× 100), and only blastocysts were used for the experiments. The blastocysts were preserved in PZM-5 [10] supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA) at 38.5 C in humidified air with 5% CO_2 until vitrification.

Vitrification and warming of embryos

The embryos were vitrified using the three different methods and warmed 20–150 days later.

In Method 1, conventional vitrification using a mini-straw (ST) was performed using 0.25-ml crystal straws (IMV Technologies, L'Aigle, France) as described previously [19] with some modifications. Briefly, the embryos were placed in a drop (80 μl) of equilibration solution consisting of PB1 medium with 11% (v/v) ethylene glycol (EG) and 0.5% BSA (11% EG solution) for 5 min at room temperature (20–25 C). After equilibration in 11% EG solution, the embryos were transferred to a vitrification solution consisting of PB1 medium supplemented with 45% EG, 7% (w/v) polyvinylpyrrolidone (MW 10,000, Sigma) and 0.5% BSA (45% EG vitrification solution) preloaded into a straw. Within 40 sec of the initial exposure of the embryos to the 45% EG vitrification solution, the straw was loaded with the solutions, as shown in Fig. 1, heat sealed and placed on a styrene foam board (5 mm thick) floating on liquid nitrogen (LN_2), and the cotton-plug part of the straw was dipped into LN_2 for a quick moment to create ice seeds in the diluent. The straw was then placed on the styrene foam board again for 3 min. After confirming that the 45% EG vitrification solution was transparent and that ice crystals had not been formed, the straw was plunged into LN_2.

After storage in LN_2 for a certain period of time, the straw was warmed in air (25 C) for 5 sec and then in a 40 C water bath for 6 sec. Immediately after warming, the straw was shaken to mix the solutions inside (i.e., first-step dilution). Two minutes after the first-step dilution, the content of the straw was expelled into a dish, and the embryos were subsequently washed by five serial transfer steps of the warmed embryos in PB1 medium supplemented with 0.5% BSA and 6%, 3%, 1.4%, 0.7% or 0% EG for 2 min each.

In Method 2, the MVC method was conducted using a Cryotop® and commercial vitrification and warming solutions (VT101 and VT102, Kitazato BioPharma, Shizuoka, Japan) by the method previously reported [3, 4]. The embryos were contracted by dehydration in an equilibration solution (ES of VT101, Kitazato BioPharma) and kept at room temperature (25 C) for 5 to 10 min until they regained their volume to some extent. The equilibrated

embryos were transferred to a vitrification solution (VS of VT101, Kitazato BioPharma) along with a minimal amount of the equilibration solution. The embryos contracted in the solution were loaded onto the tip of a Cryotop® and directly plunged into LN_2. The length of time from exposure of the embryos to the vitrification solution until storage in LN_2 was less than 60 sec.

After storage in LN_2, the embryos were warmed by immersing the Cryotop® tip into a warming solution (TS of VT102, Kitazato BioPharma) at 38.5 C. After one min, the embryos were transferred to a diluent (DS of VT102, Kitazato BioPharma) at 38.5 C for 3 min and then washed twice in a washing solution (WS of VT102, Kitazato BioPharma) for 5 min each time.

In Method 3, the PFV method was conducted using a pullulan film (1.5 mm wide, 15 mm long and 20 μm thick; Hayashibara Shoji, Okayama, Japan) attached to a stainless steel rod (Fig. 2). The embryos were kept in D-PBS with 20% FBS for 2 min, equilibrated for 4 min in D-PBS with 20% FBS supplemented with 7.5% EG, 7.5% DMSO and 0.3 M sucrose and then transferred to a vitrification solution (EDS30; D-PBS with 15% EG, 15% DMSO, 0.6 M sucrose and 20% FBS). Within 60 sec after transfer to the vitrification solution, the embryos were loaded onto the pullulan film and plunged into LN_2.

After storage in LN_2, the embryos were warmed by submerging the film successively into D-PBS supplemented with 20% FBS and 0.6 M sucrose for 2 min, D-PBS supplemented with 20% FBS and 0.3 M sucrose for 2 min and finally D-PBS with 20% FBS for 2 min.

Viability of embryos

Following warming, the embryos were cultured in TCM199 supplemented with 0.1 mM β-mercaptoethanol and 20% FBS for 24 h at 38.5 C in humidified air with 5% CO_2. The embryos forming a blastocoel were considered viable. As the non-vitrification control, embryos just recovered were also cultured for 24 h in the same medium.

Measurement of the oxygen consumption rate

The oxygen consumption rate using an embryo respirometer (HV-403, Research Institute for the Functional Peptides, Yamagata, Japan), a device developed based on a scanning electrochemical microscope, was measured for some of the embryos 30 min before vitrification and after warming by the method of Abe *et al.* [15]. Briefly, an embryo was placed into the solution for the measurement (ERAM-2, Research Institute for the Functional Peptides) on the flat bottom of a cone-shaped microwell on the plate (RAP-1, Research Institute for the Functional Peptides). A microelectrode (platinum microelectrode RAE-1, Research Institute for the Functional Peptides) was moved close to the embryo, and a voltage of –0.6 V was applied to reduce the oxygen concentration in the solution surrounding the embryo and measure the current generated as a result. The oxygen concentration gradients in the solution surrounding the embryo were measured by scanning the z-axis (i.e., horizontal direction) at a speed of 31.0 μm/sec. The average of two measurements (anterior and posterior sides of the embryo) was considered to be the oxygen consumption rate of the embryo.

Table 1. *In vitro* development of porcine embryos cryopreserved by different vitrification methods

Vitrification method	No. of embryos	
	Cultured	Survived (%)
ST	89	43 (48.3)[a]
MVC	82	58 (70.7)[b]
PFV	81	64 (79.0)[b]
Non-vitrification	54	51 (94.4)[c]

[a–c] Values with different superscripts within each column are significantly different (P<0.05).

Classification of viable and dead cells in embryos

The method of Saha and Suzuki [20] was used to compare the cell viability of the vitrified embryos. After washing with TCM199 containing 20% FBS, the embryos subjected to oxygen consumption rate measurement were warmed to 38.5 C and cultured in a staining solution for 30 min for double staining of viable and dead cells. TCM199 supplemented with 10 μg/ml bisbenzimide (Hoechst 33342, Calbiochem, San Diego, CA, USA) and 10 μg/ml propidium iodide (PI; Sigma) was used as the staining solution. The stained embryos were washed with TCM199 containing 20% FBS, placed on a slide with a small amount of TCM199 with 20% FBS and covered with a cover glass. The embryos were observed through a U-MWU filter (excitation wavelength of 365 nm, barrier filter of 400 nm) under an inverted microscope equipped with a fluorescent light source, and embryos with blue nuclei were counted as viable; those with pink nuclei were counted as dead.

Statistical analysis

The experiment was repeated three or four times for each group. A chi-square test and Fisher's exact probability test was used to compare the viability of embryos. The numbers of cells were subjected to logarithmic transformation before statistical analysis. An analysis of variance (ANOVA) was performed using the General Linear Model (GLM) procedure of SPSS (SPSS 11.5J, User's Guide, SPSS, Tokyo, Japan) followed by the Scheffe test. Linear relationships and correlation coefficients between oxygen consumption rate and the number of live cells in vitrified-warmed embryos were determined by simple regression analysis and Pearson correlation coefficient analysis, respectively. A P value of less than 0.05 was considered statistically significant.

Results

Effect of vitrification procedures on morphological changes in vitrified embryos after warming

The viabilities of embryos after vitrification and warming by the different methods are shown in Table 1. The percentage of embryos that survived in the ST group was significantly lower (P<0.05) than those in the other two vitrification groups and in the non-vitrification group. There was no significant difference in the rates between the MVC and PFV groups. Moreover, the survival rate in the non-vitrification group was significantly higher (P<0.05) than those in the other groups.

Table 2. Oxygen consumption rates of porcine embryos before and after vitrification

Vitrification method	No. of embryos examined	Oxygen consumption rate ($F \times 10^{14}$ /mol s^{-1})	
		Before vitrification	After warming*
ST	10	1.29 ± 0.17^{A}	$0.82 \pm 0.09^{a,B}$
MVC	15	1.15 ± 0.08	1.22 ± 0.08^{b}
PFV	11	0.99 ± 0.08	1.02 ± 0.09^{ab}
Non-vitrification	10	1.32 ± 0.14	–

Mean ± SEM. * Values were measured 30 min after warming. [a,b] Values with different superscripts within each column are significantly different ($P<0.05$). [A,B] Values with different superscripts within each row are significantly different ($P<0.05$).

Table 3. Cell viability of porcine embryos after vitrification

Vitrification method	No. of embryos examined	Mean cell no. in blastocyst		Ratio of live /total (%)
		Total	Live	
ST	10	70.0 ± 2.5	64.4 ± 3.2^{a}	91.9 ± 2.7^{a}
MVC	13	86.2 ± 4.3	85.9 ± 4.4^{b}	99.5 ± 0.3^{b}
PFV	9	82.3 ± 3.5	80.2 ± 4.3^{ab}	97.0 ± 1.8^{ab}
Non-vitrification	10	70.3 ± 3.7	70.3 ± 3.7^{ab}	100 ± 0^{b}

Mean ± SEM. [a,b] Values with different superscripts within each column are significantly different ($P<0.05$).

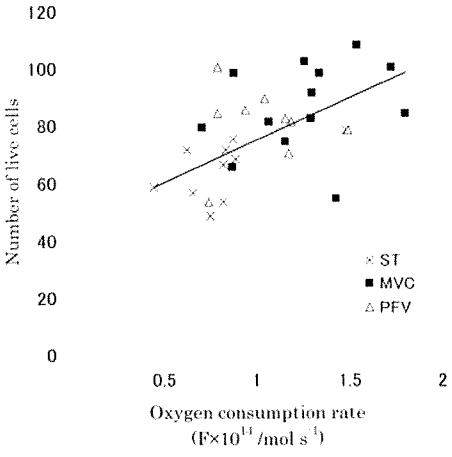

Fig. 3. Correlation between the oxygen consumption rates of vitrified-warmed embryos and number of live cells. There is a significant positive correlation between the oxygen consumption rate and the number of live cells ($P<0.01$ $r=0.496$). Regression lines are drawn to show the relationship between the oxygen consumption rates and numbers of live cells.

Effect of vitrification procedures on the oxygen consumption rates of embryos

The oxygen consumption rate of the vitrified embryos after warming was significantly higher in the MVC group ($P<0.05$) than in the ST group, but in the PFV group, it did not differ significantly from the rates in the other groups (Table 2). The oxygen consumption rate after warming was significantly lower ($P<0.05$) than that at pre-vitrification in the ST group, while there was no significant difference between these rates in the MVC and PFV groups.

Effect of vitrification procedures on cell viability in embryos

Cell viability after vitrification by the different methods is shown in Table 3. The ratio of live to total cell number was significantly lower ($P<0.05$) in the ST groups than in the MVC and non-vitrification groups. There was a positive correlation between the oxygen consumption rate and the number of live cells ($P<0.01$, $r = 0.496$) in all vitrified-warmed embryos (Fig. 3).

Discussion

The present study demonstrated that porcine blastocysts can be vitrified using a pullulan film and that viability, oxygen consumption rate and cell viability were not different from those of embryos vitrified by the MVC method using a Cryotop®.

Since vitrification uses CPAs at high concentrations to prevent intracellular ice crystal formation, their toxicity causes damage to embryos when the vitrification procedure takes a long time. To avoid this problem, several vitrification methods, such as the open-pulled straw [5], microdroplet [6] and metal mesh vitrification [7] methods, have been reported to achieve high viability by making porcine embryos very rapidly pass a critical temperature range at which embryos are injured. The viability of embryos is increased by these methods, as they have the advantages of minimizing the required amounts of CPAs by using special devises and lowering the concentration of CPAs by application of rapid cooling. The present study compared the viability of embryos in the PFV method using pullulan film with that in the MVC method, one of the ultra-rapid vitrification methods, and demonstrated that the viability was 70% or more in the PFV method, which is similar to that in the MVC method. On the other hand, the survival rate of embryos in

the ST group was significantly lower than that in the other groups. The volume of vitrification solution was smaller in the MVC and PFV groups (0.1 μl) than in the ST group (20 μl). Moreover, whereas embryos were placed into LN_2 after exposing them to LN_2 vapor in the ST group, they were directly plunged into LN_2 in the MVC and PFV groups. Thus, the difference in survival may be due to a more rapid temperature decrease in the MVC and PFV groups than in the ST group.

The vitrification procedure for embryo storage does not require a programmable freezer. However, it requires special devises and strict control of temperature and time before plunging the embryos into LN_2 because high concentrations of CPAs are used. Therefore, the number of embryos that can be vitrified in one operation has been limited. Ushijima *et al.* [4] reported that the MVC method allows simultaneous vitrification of 8 to 12 embryos. In pigs, it is desirable to cryopreserve larger numbers of embryos together because 15 to 20 embryos should be transferred into a recipient at one time. In the present study, 5 to 8 embryos could be simultaneously vitrified by using a sheet of pullulan film, and the vitrified embryos on 3 or 4 sheets of the film could be loaded together into a straw. We suggest that the PFV method to cryopreserve porcine embryos is a valuable tool for non-surgical embryo transfer in pigs, since the vitrified embryos can be directly transferred into a recipient. We have obtained normal calves after transfer of bovine blastocysts vitrified using pullulan films and warmed by one-step dilution in straws (our unpublished data). The PFV method appeared to have no adverse effect on porcine embryos in this study. If porcine embryos vitrified using pullulan films can be conveniently diluted and warmed in a straw while maintaining their viability, they may be applied to piglet production by non-surgical embryo transfer under on-farm conditions. Further technical improvements are required to enable one-step dilution in a straw.

In the present study, PFV was applied as an open method in which the solution containing embryos directly contacts LN_2. Storage of embryos in open containers in LN_2 may represent an increased potential for microbial contamination during long-term banking and the risk of disease transmission by embryo transfer [21]. To avoid contamination of pathogens, embryos collected from the same donor should be separately vitrified by the PFV method in its own sterile LN_2 and stored in its own closed containers. An in-straw dilution method after PFV may also enable decrease of the risk of contamination because storage and dilution of vitrified embryos is performed in a sealed straw.

The oxygen consumption rate of embryos vitrified by the three different methods was measured in order to assess the quality of embryos and their potential use in a non-invasive transfer method [16, 22, 23]. To our knowledge, the present study is the first to measure the oxygen consumption rate before and after vitrification of porcine embryos. The oxygen consumption rate after warming was significantly lower than the pre-vitrification levels in the ST group, while there was no significant difference between these rates in the MVC and PFV groups, thus reflecting their respective viabilities. These results were consistent with the viabilities of the embryos after vitrification and warming in these groups, supporting the validity of using the oxygen consumption rate as an index of embryo quality after vitrification and warming.

Dobrinsky *et al.* [24] have reported that success of vitrification depends on the influence of CPAs on morphological and molecular properties of cellular organelles and membranes. In the present study, cell damage of embryos was assessed by a double staining method with Hoechst 33342 and PI, which can distinguish between blue-stained live cells and pink-stained dead cells by the difference in permeability for PI, depending on damage of the cell membrane. The number of live cells in the ST group decreased significantly compared with the MVC group ($P<0.05$), and the ratio of live to total cells was reduced by 5 to 7% in the ST group compared with the other groups. This result may reflect that the survival rate of embryos after warming was markedly lower in the ST group (48.3%) than in the MVC (70.7%), PFV (79.0%) and non-vitrified groups (94.4%). Moreover, both the oxygen consumption rates of embryos just after warming and live cell numbers in the MVC group were higher than those in the ST group. In addition, there was a correlation between the oxygen consumption rate and the number of live cells in vitrified embryos after warming. Trimarchi *et al.* [22] have demonstrated that maturation of mitochondria correlates with an increase in metabolic activity, as reflected in the oxygen consumption rate, and that the oxygen consumption rate reflects the number of cells measured and their mitochondrial activity. Thus, we assumed that the number of live cells could be estimated by the oxygen consumption rate.

In the present study, no difference was found in viability and oxygen consumption rate after warming between the porcine embryos vitrified by the method using pullulan film and those obtained by the previously reported MVC method, indicating that porcine embryos can be vitrified using pullulan film. Non-surgical direct transfer of ultra-rapidly vitrified porcine embryos without using a microscope would be a useful transfer method applicable by individual farmers in the field. Therefore, further studies are desirable on the method for in-straw dilution of embryos.

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Lactate and Adenosine Triphosphate in the Extender Enhance the Cryosurvival of Rat Epididymal Sperm

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We evaluated the cryosurvival of rat epididymal sperm preserved in raffinose–modified Krebs-Ringer bicarbonate–egg yolk extender supplemented with various energy-yielding substrates (glucose, pyruvate, lactate, and ATP) and assessed the effect on sperm oxygen consumption. The incubation of sperm at 37 °C for 10 min in lactate-free extender decreased sperm motility and oxygen consumption before and after thawing compared with those of sperm in glucose- and pyruvate-free mediums. We then focused on the effect of supplementing the extender with lactate (0, 10.79, 21.58, 32.37, and 43.16 mM) and found that sperm frozen and thawed in extender supplemented with 32.37 mM lactate exhibited the highest motility. When we supplemented extender containing 32.37 mM lactate with ATP (0, 0.92, 1.85, 3.70, and 5.55 mM), sperm frozen and thawed in the extender supplemented with 1.85 mM ATP exhibited considerably higher motility and viability than those of sperm frozen and thawed in ATP-free extender. These results provide the first evidence that supplementation of the raffinose-modified Krebs–Ringer bicarbonate–egg yolk extender with 32.37 mM lactate and 1.85 mM ATP increases of number of motile sperm before freezing and enhances the cryosurvival of rat sperm. These supplements to the extender may enhance sperm cryosurvival by improving the metabolic capacity of sperm before freezing.

Abbreviations: mKRB, modified Krebs–Ringer bicarbonate.

We previously showed that freezing rat epididymal sperm in an extender of raffinose dissolved in modified Krebs–Ringer bicarbonate (mKRB) solution containing egg yolk enhances their cryosurvival of sperm as measured by viability and acrosomal integrity; this finding suggested that a mKRB-based freezing extender containing glucose, pyruvate, and lactate can protect sperm against freezing injury.[33] A possible reason for this finding is that sperm in this extender retain high metabolic capacity before freezing which, in turn, may enhance the cryosurvival of rat sperm. However, the mechanism by which the mKRB-based extender promotes the metabolic capacity and cryosurvival of rat sperm is unclear.

The process of cryopreservation imposes numerous stresses on not only the physical features of sperm but also the energy production to support motility before and after freezing, and improving energy production in frozen–thawed sperm is important for successful cryopreservation.[19] Sugars play various roles in sperm extender solution, including providing an energy substrate for sperm during cooling and acting as a cryoprotectant.[1] The beneficial effects of adding glucose to the extender on the viability of frozen–thawed sperm have been reported for various species;[2] this nutritional effect may involve the synthesis and provision of ATP through the glycolytic pathway to provide the energy required for sperm motility. Therefore, glucose may play a key role in generating energy in motile sperm and preventing freezing damage.

The addition of an exogenous substrate (such as lactate) improved the sperm motility characteristics of cattle,[11] boars,[14,21] and rabbits.[29] A previous study[9] showed that a shuttle involving the redox couple lactate–pyruvate and lactate dehydrogenase isozyme C_4 is active in rat and rabbit mitochondria but not in mouse mitochondria. This finding suggests that sperm from various species, including rats, may find lactate a suitable substrate for maintaining the energy production and consumption as well as oxygen consumption. However, which of the major biochemical pathways—glycolysis or oxidative phosphorylation—is involved in supplying energy to mobilize sperm has been a long-lasting debate, because the pathway of energy production is species-specific.[8,28,32] Regardless of the identity of the pathway involved in energy supply, the development of an appropriate freezing extender likely would improve sperm motility and survival during cryopreservation through enhancement of the metabolic capacities of sperm. However, no previous studies have reported on the optimal components, especially the energy substrates, of a cryodiluent for the freezing of rat sperm.

Sperm may attain access to eggs by mobilizing metabolic energy production in the form of ATP to drive motility.[30] ATP is hydrolyzed by the dynein adenosine triphosphatase, which converts the chemical energy of ATP into mechanical energy used for the movement of sperm.[3,15] ATP can have several downstream effects leading to improvement in the motility of sperm by means of an increase in the calcium level.[10,17,18,20,25,27] Moreover, the amount of ATP required for metabolic energy is higher in the cytosol than the mitochondria.[16] Therefore, sup-

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plementation of the freezing extender with exogenous ATP may improve the cryosurvival of rat epididymal sperm.

Here we evaluated the cryosurvival and parameters of mitochondrial activity, including oxygen consumption, of rat sperm diluted in raffinose–mKRB–egg yolk extender supplemented with various energy-yielding substrates, including glucose, pyruvate, lactate, and ATP. In addition, we identified the optimal energy substrates and other components of a cryodiluent for the freezing of rat sperm.

Materials and Methods

Principles of laboratory animal care were followed during this study, and all procedures were conducted in accordance with guidelines of the Ethics Committee for Care and Use of Laboratory Animals for Research of the Graduate School of Agricultural Science (Tohoku University, Japan). Wistar rats were used throughout the experiments. Animals were kept in polycarbonate cages (25 × 40 × 20 cm) under controlled conditions with lights on at 0800 and off at 2000. They were given food and tap water ad libitum.

Preparation of the raffinose–mKRB–egg yolk extender. The basic extender used in this study was the raffinose–mKRB–egg yolk freezing solution defined previously;[33] it comprised 0.1 M raffinose (Sigma, St Louis, MO), 94.6 mM NaCl (Wako Pure Chemical Industries, Osaka, Japan), 4.78 mM KCl (Wako), 1.71 mM $CaCl_2 \cdot 2H_2O$ (Wako), 1.19 mM $MgSO_4 \cdot 7H_2O$ (Wako) 1.19 mM KH_2PO_4 (Wako), 25.07 mM $NaHCO_3$ (Wako), 21.58 mM sodium DL-lactate (Sigma), 0.5 mM sodium pyruvate (Wako), 5.56 mM glucose (Wako), 50 µg/mL streptomycin (Sigma), and 75 µg/mL penicillin (Sigma); egg yolk was separated from the albumin, and 20% (v:v) egg yolk was added to the raffinose–mKRB solution. Egg yolk lipids were solubilized by adding 0.04% (w:v) SDS (Wako) to the solution. The solution was centrifuged twice at 7000 × *g* for 30 min. The pH of the solution was adjusted to 7.3 with HCl and its osmotic pressure to 400 mOsm. The supernatant was aspirated and filtered through a 0.45-µm membrane filter (Sartorius, Goettingen, Germany).

Evaluation of sperm motility parameters. Sperm motility parameters were assessed by using a sperm motility analysis system (version 1.0, Kashimura, Tokyo, Japan) and a 10-µm deep Makler chamber (Sefi Medical Instruments, Haifa, Israel); the protocol was described previously.[6] At least 100 sperm and 5 fields were assessed by the sperm motility analysis system for each treatment group. The following parameters were assessed in this study: motility (%), straight line velocity (µm/s), curvilinear velocity (µm/s), amplitude of lateral head displacement (µm), and beat cross frequency (Hz).

Evaluation of sperm acrosome integrity. The acrosomal integrity of fresh and frozen–thawed sperm was assessed by staining with FITC-conjugated peanut agglutinin (Wako) according to the procedure described previously.[33]

Collection of rat epididymal sperm. Both caudae epididymides were excised from 24 sexually mature male Wistar rats older than 15 wk. The excised epididymis was rinsed and carefully blotted free of blood and adipose tissues. A small part of the caudae epididymides tract was excised with fine scissors. The droplet of sperm that welled up was transferred to a 1.5-mL microfuge tube containing 1 mL of freezing medium at 37 °C. After 5 min, the solution was examined macroscopically to verify that sperm were dispersed adequately.

Cryopreservation and thawing. ***Experiment 1a.*** In this experiment, we investigated the effect of the substrates glucose, pyruvate, and lactate in raffinose–mKRB–egg yolk freezing extender on the motility characteristics of fresh sperm after collection and frozen–thawed sperm. Sperm from both the caudae epididymides from 3 rats were used in this experiment. Immediately after collection, aliquots of sperm were exposed to the following 5 solutions: raffinose–mKRB–egg yolk extender containing the substrates glucose, pyruvate, and lactate (control); glucose-free extender; pyruvate-free extender; lactate-free extender; and substrate-free extender. The osmotic pressure of these solutions was adjusted to 400 mOsm with sucrose (Wako) and the pH to 7.3 with HCl. Each sperm suspension was incubated at 37 °C for 5 min to allow the sperm to disperse, and the sperm concentration and motility parameters then were evaluated by the sperm motility analysis system. The sperm were processed and frozen by using a modification of a previously published protocol.[33] The diluted sperm samples were cooled at 5 °C for 90 min. The sperm samples were further diluted 1:1 with each extender containing 1.5% of a commercial cryoprotectant (Equex STM, Nova Chemical Sales, Scituate, MA) to obtain a sperm concentration of 5×10^6 sperm/mL and then were equilibrated at 5 °C for 30 min before freezing. Afterward, the samples were loaded into standard 0.5-mL straws and the straws were heat-sealed. The straws were placed in liquid nitrogen vapor for 10 min, plunged into liquid nitrogen (–196 °C), and stored for 3 d at this temperature. The straws were thawed rapidly by holding them in water (37 °C) for 10 s. The sperm were transferred to a 1.5-mL microfuge tube and incubated at 37 °C for 5 min, after which motility parameters after thawing were assessed by using the sperm motility analysis system. The acrosome status of frozen–thawed sperm was assessed by staining with FITC-conjugated peanut agglutinin.[1]

Experiment 1b. Building on the results of Experiment 1a, this experiment was conducted to analyze the characteristics of fresh and frozen–thawed sperm in the raffinose–mKRB–egg yolk extender containing various concentrations of lactate. Sperm from both caudae epididymides from 3 rats were used. Aliquots of sperm were suspended in 1 mL raffinose–mKRB–egg yolk extender containing 0, 10.79, 21.58, 32.37, or 43.16 mM lactate; all of these solutions had an osmotic pressure of 400 mOsm and a pH of 7.3, except the solution containing 43.16 mM lactate (430 mOsm and pH 7.3). The procedures followed for cryopreservation and evaluation of sperm were the same as described for experiment 1a.

Experiment 1c. This experiment was designed to compare the cryosurvival of rat sperm frozen in raffinose–mKRB–egg yolk extender solution containing 32.37 mM lactate supplemented with various concentrations of ATP. Both caudae epididymides from 3 male rats were used in this experiment. After collection, sperm was divided into 5 aliquots and suspended in 1 mL of extender solution containing 32.37 mM lactate and 0, 0.92, 1.85, 3.70, or 5.55 mM ATP (400 mOsm and pH 7.3). The freezing protocol and evaluation of sperm were the same as described previously. For the evaluation of sperm viability, frozen–thawed sperm samples were incubated in a water bath at 37 °C for 5 min. For each treatment, 3 samples were evaluated after 1, 2, and 3 h of incubation to determine sperm motility, straight-line velocity, curvilinear velocity, and amplitude of lateral head displacement.

Measurement of oxygen consumption of sperm. ***Experiment 2a.*** The aim of this experiment was to assess the effect of substrates in the raffinose–mKRB–egg yolk medium on the mitochondrial activity of sperm. Sperm was collected from 5 mature male rats and extended in substrate-free raffinose–mKRB–egg yolk medium at 37 °C. The sample was incubated for 5 min to allow the sperm to disperse and then equal volumes were resuspended in each of the following solutions: raffinose–

mKRB–egg yolk medium containing 11.12 mM glucose, 1 mM pyruvate, and 43.16 mM lactate (control); glucose-free control (containing pyruvate and lactate); pyruvate-free control (containing glucose and lactate); lactate-free control (containing glucose and pyruvate); and substrate-free raffinose–mKRB–egg yolk medium. The final concentrations of the various substrates in medium containing sperm were 5.56 mM glucose, 0.5 mM pyruvate, and 21.58 mM lactate. The osmotic pressure of these solutions was adjusted to 400 mOsm with sucrose and the pH to 7.3 with HCl. The oxygen consumption rates of the sperm were measured by using Clark-type oxygen electrodes (Rank Brothers, Cambridge, UK) maintained at 37 °C for 10 min and calibrated with air-saturated water at 37 °C, which was assumed to contain 406 nmol oxygen/mL.[26] A sperm sample in a volume of 1 mL was suspended in the reaction chamber by stirring carefully to prevent the addition of any external air. The final concentration of sperm in the incubation chamber was approximately 1×10^7 sperm/mL. Data were acquired by using a commercial software program (LabChart version 5.2, AD Instruments, Castle Hill, Australia). The oxygen consumed by the sperm was calculated as:[26]

> Oxygen concentration (nmol oxygen/mL) = oxygen (U) × oxygen concentration of air-saturated water (that is, 406 nmol oxygen/mL) ÷ oxygen full-chart span (U)

The rate of oxygen consumption by sperm was expressed as nmol/min/1×10^7 sperm.

Experiment 2b. The effect of various concentrations of lactate in the raffinose–mKRB–egg yolk extender on the oxygen uptake of sperm was analyzed. Sperm were collected from the caudae epididymides of 5 rats and suspended in lactate-free raffinose–mKRB–egg yolk medium and then incubated for 5 min at 37 °C. Equal volumes of raffinose–mKRB–egg yolk medium containing 0, 21.58, 43.16, 64.74, and 86.32 mM lactate were diluted with lactate-free raffinose–mKRB–egg yolk medium, resulting in solutions with final lactate concentrations of 0, 10.79, 21.58, 32.37, and 43.16 mM, respectively. The osmotic pressure and the pH of all these solutions were adjusted to 400 mOsm and 7.3, respectively, except the solution containing 43.16 mM lactate (430 mOsm and pH 7.3). The oxygen consumption of each sperm suspension was determined in relation to air-saturated medium as described for experiment 2b.

Experiment 2c. The effect of adding ATP to raffinose–mKRB–egg yolk medium containing 32.37 mM lactate on the rate of oxygen consumption of sperm was examined. Sperm from 5 rats were flushed the sperm out by using the medium, and then the suspensions in ATP-free raffinose–mKRB–egg yolk medium were incubated for 5 min at 37 °C. Each treated sample was placed in raffinose–mKRB–egg yolk medium containing 0, 1.84, 3.70, 7.4, or 11.1 mM ATP. Subsequently, equal volumes of extended sperm were added to these solutions, resulting in solutions with final ATP concentrations of 0, 0.92, 1.85, 3.70, and 5.55 mM (400 mOsm and pH 7.3). The oxygen consumption of the sperm was determined as described for experiment 2a.

Statistical analysis. The data were subjected to ANOVA and the Fisher protected least-significant difference post hoc test (StatView, Abacus Concepts, Berkeley, CA). All data are expressed as mean ± SEM. A P value of less than 0.05 indicated statistical significance.

Results

Effect of various substrates in raffinose–mKRB–egg yolk extender on fresh and frozen–thawed sperm (experiment 1a). The first experiment in this series was aimed at assessing the effect of various energy-yielding substrates in the raffinose–mKRB–egg yolk extender on the motility characteristics of fresh and frozen–thawed sperm. The motility of sperm added to the medium without the substrates glucose, pyruvate, and lactate was significantly ($P < 0.05$) lower than that of sperm added to the medium containing all 3 of these substrates (control; Table 1); this result was obtained from both fresh and frozen–thawed sperm. In contrast, the sperm motility and motion parameters did not differ significantly between fresh and frozen–thawed sperm when glucose-free and pyruvate-free solutions were used. The medium that contained glucose, pyruvate, and lactate resulted in the highest motility of frozen–thawed sperm. The percentage of intact acrosomes did not differ significantly among sperm treated with the various extenders for both fresh and frozen–thawed sperm (Table 1).

Effect of lactate in raffinose–mKRB–egg yolk extender on fresh and frozen–thawed sperm (experiment 1b). According to the results of experiment 1a, lactate was the most effective agent for increasing the motility of both fresh and frozen–thawed sperm. We therefore investigated the effect of adding lactate at 0, 10.79, 21.58, 32.37, and 43.16 mM to the raffinose–mKRB–egg yolk medium on the motility of sperm. Sperm diluted in lactate-free extender showed significantly ($P < 0.05$) lower motility than did sperm diluted in extender containing 21.58 or 32.37 mM lactate (Table 2). The data revealed that sperm frozen in the raffinose–mKRB–egg yolk extender containing 32.37 mM lactate showed significantly ($P < 0.05$) higher motility after thawing than did sperm frozen in substrate-free extender. The proportion of sperm with intact acrosomes either before or after thawing did not differ significantly among extenders containing 0, 10.79, 21.58, 32.37, or 43.16 mM lactate.

Effect of ATP in raffinose–mKRB–egg yolk extender containing 32.37 mM lactate on fresh and frozen–thawed sperm (experiment 1c). The effect of adding 0, 0.92, 1.85, 3.70, or 5.55 mM ATP to raffinose–mKRB–egg yolk extender containing 32.37 mM lactate on the cryosurvival of the sperm are summarized in Table 3. Sperm frozen in extender containing 32.37 mM lactate and 1.85 mM ATP exhibited significantly ($P < 0.05$) higher motility than that of sperm frozen in ATP-free extender. The sperm frozen and thawed in extender supplemented with 1.85 mM ATP maintained significantly ($P < 0.05$) higher motility throughout the 3-h incubation at 37 °C than did sperm frozen and thawed in the ATP-free extender (Figure 1). The addition of ATP to the extender increased the proportion of intact acrosomes in after collected sperm, and among all concentrations of ATP tested, the percentage of intact acrosomes was highest at 1.85 mM ATP. Similar results were obtained for the acrosome status of frozen–thawed sperm.

Effect of glucose, pyruvate, and lactate in raffinose–mKRB–egg yolk medium on the oxygen consumption of sperm (experiment 2a). In the next series of experiments, we examined the effect of the substrates glucose, pyruvate, and lactate in the raffinose–mKRB–egg yolk medium on the rate of oxygen consumption of sperm. Incubation of the sperm suspension with lactate-free medium resulted in a significant ($P < 0.05$) decline in the rate of oxygen consumption during incubation as compared with the incubation of the sperm suspension with medium containing glucose, pyruvate, and lactate (Figure 2). The oxygen consumption of sperm in medium lacking any added substrates tended to be decreased compared with that of sperm in the complete

Table 1. Effect of the substrates glucose, pyruvate, and lactate in raffinose–mKRB–egg yolk extender on fresh and frozen–thawed sperm

	Sperm characteristics	Control	–Glucose	–Pyruvate	–Lactate	Substrate-free
Fresh sperm	Motility (%)	78.2 ± 8.0	70.8 ± 2.6	67.3 ± 8.0	54.9 ± 6.1	44.8 ± 3.9[a]
	VSL (μm/s)	17.4 ± 1.2	16.5 ± 3.3	10.9 ± 1.4	15.4 ± 1.0	11.1 ± 1.9
	VCL (μm/s)	124.2 ± 3.1	115.1 ± 8.6	109.8 ± 1.9	108.3 ± 2.9	102.4 ± 4.1
	ALD (μm)	6.9 ± 0.1	6.5 ± 0.5	6.5 ± 0.2	6.5 ± 0.1	5.8 ± 0.3
	BCF (Hz)	22.2 ± 0.8	23.3 ± 1.8	25.4 ± 1.6	23.4 ± 1.0	29.0 ± 0.6
	Acrosomal integrity (%)	84.5 ± 2.9	76.2 ± 1.9	79.6 ± 4.9	77.1 ± 2.4	81.4 ± 4.4
Frozen–thawed sperm	Motility (%)	21.5 ± 1.4	23.2 ± 0.9	19.8 ± 1.3	13.8 ± 2.4[a]	13.7 ± 1.5[a]
	VSL (μm/s)	3.9 ± 0.4	3.2 ± 0.5	3.9 ± 0.4	3.9 ± 0.3	4.4 ± 0.8
	VCL (μm/s)	85.4 ± 8.7	71.8 ± 4.7	89.2 ± 13.2	77.8 ± 5.5	69.3 ± 7.4
	ALD (μm)	4.2 ± 0.5	3.3 ± 0.2	4.1 ± 0.5	3.8 ± 0.2	3.2 ± 0.5
	BCF (Hz)	32.2 ± 3.7	31.3 ± 1.6	36.2 ± 1.8	34.1 ± 0.9	31.7 ± 2.5
	Acrosomal integrity (%)	68.1 ± 4.0	71.3 ± 5.8	70.0 ± 2.0	64.1 ± 5.3	70.6 ± 2.3

ALD, amplitude of lateral head displacement; BCF, beat cross frequency; VCL, curvilinear velocity; VSL, straight-line velocity.
Data are presented as mean ± SEM (n = 3).
[a]Value significantly ($P < 0.05$) different from control value.

Table 2. Effect of lactate in raffinose–mKRB–egg yolk extender on fresh and frozen–thawed sperm

		Lactate concentration (mM)				
	Sperm characteristics	0	10.79	21.58	32.37	43.16
Fresh sperm	Motility (%)	47.4 ± 4.9	46.3 ± 13.2	61.3 ± 2.1	67.5 ± 3.8[a]	55.1 ± 3.7
	VSL (μm/s)	12.5 ± 1.7	8.8 ± 1.0	16.5 ± 2.6	12.3 ± 1.2	9.8 ± 3.0
	VCL (μm/s)	98.3 ± 6.3	86.0 ± 2.7	94.6 ± 10.0	92.9 ± 3.1	95.9 ± 6.5
	ALD (μm)	5.3 ± 0.4	5.3 ± 0.3	4.8 ± 0.6	4.7 ± 0.3	5.1 ± 0.3
	BCF (Hz)	26.4 ± 1.4	24.7 ± 1.3	22.5 ± 0.8	24.5 ± 0.8	25.6 ± 1.3
	Acrosomal integrity (%)	75.9 ± 0.5	71.7 ± 4.0	73.6 ± 5.9	75.1 ± 0.5	71.8 ± 1.8
Frozen–thawed sperm	Motility (%)	11.3 ± 2.2	17.5 ± 3.5	19.7 ± 2.8	22.3 ± 4.0[a]	12.6 ± 4.3
	VSL (μm/s)	3.5 ± 0.6	2.8 ± 0.3	3.6 ± 0.2	3.8 ± 0.1	4.9 ± 0.5
	VCL (μm/s)	78.6 ± 8.2	75.0 ± 3.5	78.5 ± 4.2	95.0 ± 14.1	126.9 ± 5.1[a]
	ALD (μm)	3.4 ± 0.6	3.3 ± 0.1	3.5 ± 0.2	4.5 ± 0.6	5.5 ± 0.4[a]
	BCF (Hz)	44.8 ± 1.5	36.7 ± 1.8	39.8 ± 0.8	36.5 ± 2.3	42.9 ± 2.1
	Acrosomal integrity (%)	69.3 ± 2.9	71.6 ± 0.4	69.0 ± 0.2	69.2 ± 0.2	61.5 ± 1.8

ALD, amplitude of lateral head displacement; BCF, beat cross frequency; VCL, curvilinear velocity; VSL, straight-line velocity.
Data are presented as mean ± SEM (n = 3).
[a]Value significantly ($P < 0.05$) different from control value.

medium. In contrast, oxygen consumption did not differ significantly between sperm in glucose- or pyruvate-free media and those in the medium containing glucose, pyruvate, and lactate.

Effect of lactate in raffinose–mKRB–egg yolk medium on the oxygen consumption of sperm (experiment 2b). The respiration capacity of sperm was tested after their incubation in a lactate-free raffinose–mKRB–egg yolk medium or in a medium supplemented with 10.79, 21.58, 32.37, or 43.16 mM lactate (Figure 3). Oxygen uptake was significantly ($P < 0.05$) higher in sperm incubated in medium containing 32.37 mM lactate than in sperm incubated in lactate-free medium.

Effect of ATP in the raffinose–mKRB–egg yolk extender containing 32.37 mM lactate on the oxygen consumption of sperm (experiment 2c). This experiment evaluated the influence of supplementation of the raffinose–mKRB–egg yolk medium containing 32.37 mM lactate with various concentrations of ATP (0, 0.92, 1.85, 3.70, and 5.55 mM) on the oxygen consumption of sperm during incubation at 37 °C for 10 min (Figure 4). When the medium was supplemented with 1.85 mM ATP, the rate of oxygen consumption tended to be increased compared with that in ATP-free medium, but difference is not significant.

Discussion

The present study demonstrated that an extender of raffinose–mKRB–egg yolk containing 32.37 mM lactate enhanced the metabolic capacity and survival of rat sperm after cryopreservation. The cryosurvival of rat sperm was further improved by the addition of 1.85 mM exogenous ATP to the freezing extender.

When the oxidizable substrate lactate was not added to the raffinose–mKRB–egg yolk extender, the motility, viability, and rate of oxygen consumption decreased considerably in both fresh and frozen–thawed sperm. In contrast, sperm frozen and thawed in extender supplemented with 32.37 mM lactate exhibited higher motility than those frozen and thawed in lactate-free extender. This finding indicates that exogenous lactate in the freezing extender is a potent inducer that enhances the oxygen consumption of rat sperm and their motility after collection and freezing–thawing.

Table 3. Effect of ATP in raffinose–mKRB–egg yolk extender containing 32.37 mM lactate on fresh and frozen–thawed sperm

		ATP concentration (mM)				
		0	0.92	1.85	3.70	5.55
Fresh sperm	Motility (%)	74.7 ± 1.8	72.8 ± 8.1	79.2 ± 3.3	73.8 ± 6.6	55.6 ± 4.2[a]
	VSL (μm/s)	9.1 ± 1.7	10.1 ± 0.5	14.7 ± 3.3	7.9 ± 0.6	8.4 ± 1.4
	VCL (μm/s)	110.2 ± 9.9	116.9 ± 8.5	123.6 ± 5.2	101.5 ± 8.8	104.9 ± 7.9
	ALD (μm)	6.0 ± 0.1	7.6 ± 0.6	6.3 ± 0.6	6.8 ± 1.0	5.7 ± 0.4
	BCF (Hz)	29.9 ± 2.8	34.0 ± 1.7	29.7 ± 2.7	32.9 ± 2.6	31.2 ± 1.5
	Acrosomal integrity (%)	75.5 ± 6.3	78.9 ± 6.5	83.2 ± 1.6	82.5 ± 7.2	77.1 ± 7.1
Frozen–thawed sperm	Motility (%)	20.6 ± 0.3	24.6 ± 0.9	35.3 ± 1.3[a]	26.9 ± 1.4	25.7 ± 7.2
	VSL (μm/s)	6.0 ± 1.2	5.0 ± 0.2	4.1 ± 0.6	4.4 ± 0.6	3.0 ± 0.4
	VCL (μm/s)	99.0 ± 8.3	89.3 ± 4.5	84.5 ± 4.1	85.8 ± 2.7	80.9 ± 3.9
	ALD (μm)	5.2 ± 0.8	4.4 ± 0.4	4.3 ± 0.1	4.3 ± 0.1	3.4 ± 0.1
	BCF (Hz)	37.1 ± 2.0	34.4 ± 0.6	35.0 ± 1.6	34.6 ± 5.0	37.2 ± 2.2
	Acrosomal integrity (%)	67.7 ± 2.3	68.4 ± 8.8	70.6 ± 4.6	61.7 ± 3.5	66.5 ± 3.8

ALD, amplitude of lateral head displacement; BCF, beat cross frequency; VCL, curvilinear velocity; VSL, straight-line velocity.
Data are presented as mean ± SEM (n = 3).
[a]Value significantly ($P < 0.05$) different from control value.

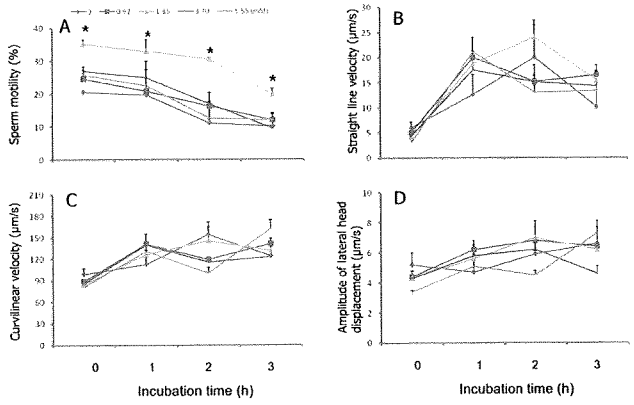

Figure 1. Effect of ATP in raffinose–mKRB–egg yolk medium containing 32.37 mM lactate on the (A) motility, (B) straight line velocity, (C) curvilinear velocity, and (D) amplitude of lateral head displacement of frozen–thawed sperm during incubation at 37 °C for 3 h. Data are presented as mean ± SEM (n = 3). *, Value significantly ($P < 0.05$) different from control value.

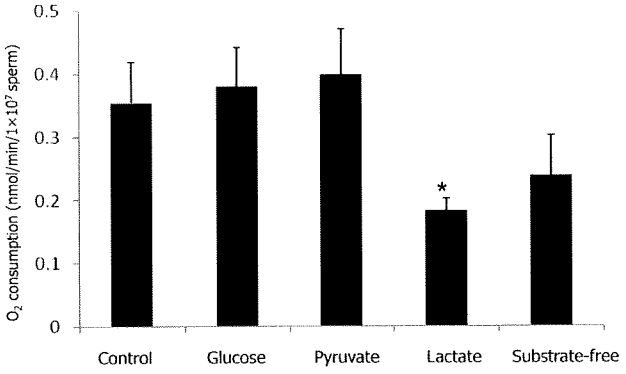

Figure 2. Effect of glucose, pyruvate, and lactate in raffinose–mKRB–egg yolk medium on the oxygen consumption of fresh sperm during incubation at 37 °C for 10 min. Data are presented as mean ± SEM (n = 5). *, Value significantly ($P < 0.05$) different from control value.

The sperm-specific enzyme lactate dehydrogenase isozyme C_4 is located in the cytosol and the matrix of the mitochondria in the midpiece of rat sperm. Further, a study[9] has revealed that both a shuttle involving the redox couple lactate–pyruvate and lactate dehydrogenase isozyme C_4 are active in rat sperm mitochondria. In another study,[12] the lactate concentration in oviductal fluids was 10-fold higher than the glucose concentration, and the lactate concentration in the uterine fluids was 15-fold higher than the glucose concentration during the murine estrous cycle. Therefore, it is very likely that lactate is used by rat sperm as an essential substrate to maintain highly regulated ATP production and dissipation: lactate in the cytosol and mitochondrial matrix is oxidized to pyruvate by mitochondrial lactate dehydrogenase isozyme C_4, and pyruvate is oxidized through the Krebs cycle and electron transport chain.[4,5,23,24] To our knowledge, our findings are the first evidence showing that rat sperm can use exogenous lactate in the cryodiluent as an essential substrate to maintain highly regulated metabolic capacity and that this lactate acts as an energy substrate for mitochondria to the mobilization of fresh and frozen–thawed sperm.

Mitochondria, the site of ATP generation due to oxidative phosphorylation, are localized solely in the midpiece of sperm.[22] The oxidative production of ATP through the Krebs cycle is an essential function of the midpiece mitochondria for motility.[31] The mitochondrial biochemical pathways of oxidative phosphorylation are 15 times more efficient than is anaerobic glycolysis for ATP production.[7,28] These findings also support our arguments that the energy production and dissipation in rat sperm are highly dependent on the mitochondria.

The present study showed that supplementation of raffinose–mKRB–egg yolk extender with 32.37 mM lactate and 1.85 mM exogenous ATP considerably increases sperm motility before freezing, thus improving the survivability of sperm after cryopreservation. Exogenous ATP in the freezing medium may be responsible for the generation of multiple metabolic signals that appear to be related to the sperm motility through a rise in calcium levels;[10,17,18,20,25,27] this reaction increases de novo ATP synthesis before freezing and may contribute to the remobilization of sperm after freezing–thawing. The motility of ram sperm was restored by exogenous ATP that crossed plasma membrane when the membrane was damaged by cryopreservation.[13] In light of that finding,[13] we cannot discount that our result is caused by the facultative transport of ATP across plasma membrane because of damage during freezing, thereby allow-

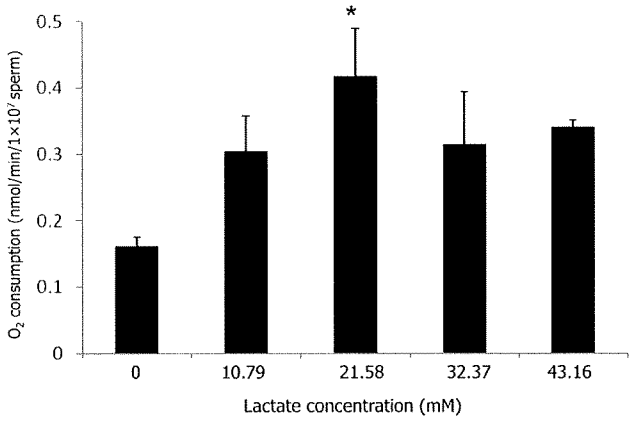

Figure 3. Effect of lactate in raffinose–mKRB–egg yolk medium on oxygen consumption of fresh sperm during incubation at 37 °C for 10 min. Data are presented as mean ± SEM (n = 5). *, Value significantly ($P < 0.05$) different from control value.

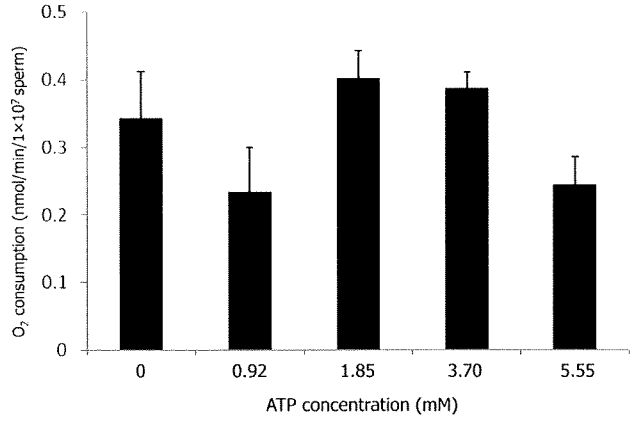

Figure 4. Effect of ATP in raffinose–mKRB–egg yolk medium containing 32.37 mM lactate on oxygen consumption of fresh sperm during incubation at 37 °C for 10 min. Data are presented as mean ± SEM (n = 5). *, Value significantly ($P < 0.05$) different from control value.

ing substrates to directly access ATP and allowing adenosine triphosphatase to use ATP directly to generate energy for the mobilization of rat sperm.

In conclusion, the current study demonstrated that the addition of lactate and ATP to the raffinose–mKRB–egg yolk extender before freezing increases the number of motile sperm and mediates the energy-dependent synthetic processes of rat epididymal sperm. In turn, these effects may increase the cryosurvival of rat sperm. Further investigation of species-specific differences in the energy-dependent synthetic processes in sperm may prove valuable in defining the ideal components of a cryodiluent, which interact to regulate the cryosurvival of rat sperm, and in clarifying the adaptations needed for cryopreservation of sperm from other species.

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CASE REPORT

A birth from the transfer of a single vitrified-warmed blastocyst using intracytoplasmic sperm injection with calcium ionophore oocyte activation in a globozoospermic patient

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Objective: To present the effectiveness of diagnostic heterologous intracytoplasmic sperm injection (ICSI), mouse oocyte activation test (MOAT), and ICSI combined with assisted oocyte activation (AOA) in a globozoospermic patient.
Design: A case report.
Setting: A private IVF center, Japan.
Patient(s): A patient with globozoospermia.
Intervention(s): MOAT in a mouse and ICSI combined with AOA in a human.
Main Outcome Measure(s): Ultrastructure, MOAT, fertilization, and pregnancy.
Result(s): The transmission electron micrographs showed 100% round-headed spermatozoa lacking an acrosome. MOAT showed that the fertilization rate was 68.4% (13/19) when AOA was used but 0% (0/19) when AOA was not used. After the diagnosis of globozoospermia and sperm-related activation deficiency, 17 human mature oocytes were activated with calcium ionophore after ICSI was performed. The fertilization rate was 88.2% (15/17), and 11 blastocysts were cryopreserved using the vitrification method to prevent severe ovarian hyperstimulation syndrome. A single vitrified-warmed blastocyst was transferred. A gestational sac with fetal heart movements was recognized, and a healthy boy weighing 3180 g was born at 40 weeks of gestation by cesarean section without any congenital abnormality.
Conclusion(s): MOAT allows discrimination between sperm- and oocyte-related fertilization failures and shows the effectiveness of AOA. (Fertil Steril® 2009;91:931.e7–e11. ©2009 by American Society for Reproductive Medicine.)

Key Words: Globozoospermia, calcium ionophore A23187, strontium chloride ($SrCl_2$), ICSI, diagnostic heterologous ICSI, assisted oocyte activation (AOA), round-headed spermatozoa, lack of an acrosome

Globozoospermia is a rare (incidence <0.1% in male infertile patients) form of teratozospermia, mainly characterized by round-headed spermatozoa that lack an acrosome. It originates from a disturbed spermiogenesis, which is known to be genetic. These sperm lack acrosomal membranes and acrosin contents, so they are unable either to penetrate the zona pellucida of an oocyte or to fuse with the oolemma in vivo or in vitro. However, intracytoplasmic sperm injection (ICSI) has opened up new possibilities to couples with male factor infertility caused by globozoospermia (1–9). However, ICSI with globozoospermic cells is generally less successful compared with typical ICSI (10). Rybouchkin et al. (11) discovered that fertilization was improved when a calcium ionophore was used as well. They suggested that the sperm-associated oocyte-activating factor that normally causes the Ca^{2+} flux required for fertilization might be absent or down-regulated in globozoospermic sperm. We report a successful case of pregnancy and delivery from a transfer of a single vitrified-warmed blastocyst after performing ICSI and assisted oocyte activation (AOA) in a patient with globozoospermia and asthenozoospermia.

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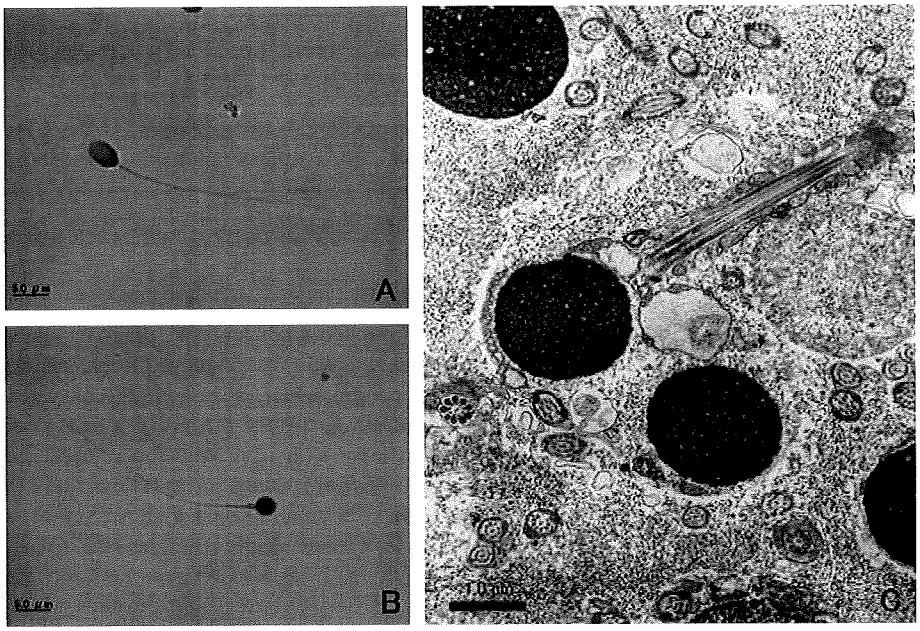
CASE REPORT

Informed consent was obtained from the couple before the study. The couple, with primary infertility of 2 years' duration, was healthy and had no physical issues except for the

FIGURE 1

Round-headed sperm morphology in a globozoospermic patient. (**A**) Normal sperm morphology, control aspects by light microscope. (**B**) Round-headed sperm morphology of a patient by light microscope. (**C**) Round-headed sperm morphology of a patient by electron microscope.



Kyono. Birth from a globozoospermic patient. Fertil Steril 2009.

husband's semen characteristics. A Kruger test showed 100% round-headed sperm with abnormal morphology. We diagnosed the husband as having globozoospermia by electron microscopy. Since we confirmed the efficacy of AOA by mouse oocyte activation test (MOAT), ICSI with AOA was performed for human oocytes.

MATERIALS AND METHODS

Patient History (Anamnesis)

Before this study, we obtained informed consent from the couple and approval from the Institutional Review Board for this study. A 29-year-old woman and her 30-year-old husband presented with primary infertility of 2 years' duration. The couple was healthy and had no physical issues except for the husband's semen aspects. The fertility of the wife was completely normal. Semen analysis showed normal values in volume (2.0 mL), concentration (38 $\times$ 10^6/mL), and motility (39%), which proved asthenozoospermia, and the analysis also showed 100% round-headed sperm with abnormal morphology on light and electron microscopy (Fig. 1). The karyotypes of the couple were 46,XX (wife) and 46,XY (husband) on peripheral lymphocytes.

Fixation and Observation for Electron Microscopy

Sperm were processed for transmission electron microscopy using the method described elsewhere (12), by which they were collected with centrifugation and fixed in chilled 2.5% glutaraldehyde (Wako; Osaka, Japan) solution in 0.1 M phosphate buffer, pH 7.4. After they were washed with chilled 0.1 M phosphate buffer, the sperm were postfixed in chilled 1% osmium teroxide (Taab Laboratories Equipment Ltd., Berkshire, UK) in 0.1 M phosphate buffer, dehydrated in a series of graded ethanol, and embedded in epoxy resin (Taab Laboratories Equipment Ltd.). Ultrathin sections were cut with a diamond knife using an ultramicrotome (Reichert Ultracuts, Leica; Heerbrugg, Switzerland), stained with uranyl acetate and lead citrate, and examined by a transmission electron microscope (IEM-1210, Jeol; Tokyo, Japan).

MOAT

Preparation of mouse oocytes Mature B6D2F1 female mice, 8–12 weeks of age, were superovulated by IP injections of 5 IU pregnant mare serum gonadotropin (PMSG) followed by the administration of 5 IU hCG 48 hours later. The mouse oocytes were collected from the oviducts of the females 14–16 hours after the hCG injection. The oocytes were freed from cumulus cells by pipetting in a Hepes-human follicular fluid medium (Hepes-HFF99; Fuso Pharmaceutical Industries, Osaka, Japan) supplemented with 10% synthetic serum substance (SSS; Irvine Scientific, Santa Ana, CA) and 60 IU/mL bovine testicular hyaluronidase (Sigma Chemical Co., St. Louis) at 37°C. These oocytes were rinsed and kept in an HFF