(GnRH, Danazol など) が追加される場合があり，妊孕性よりも疼痛治療を優先する場合にはGnRH製剤の術後投与が術後の再発率を下げる可能性が指摘されている．しかし，術前のGnRH投与は病巣部位の線維化が強くなり，内膜症病巣の切除が困難となることがあり注意が必要である．

根治的切除後（子宮全摘および卵巣切除後）にも残存した一部の卵巣や，子宮内膜症組織から子宮内膜症が進展する症例もまれにあり，根治術後のエストロゲン剤投与を行わざるをえない場合は細心の注意が必要である．難治性子宮内膜症病変の治療にaromatase inhibitorの使用も試みられていて，閉経後の骨盤腔内に子宮内膜症が進展した症例に有効であったとの報告もあり，aromatase inhibitorは今後，子宮内膜症の治療薬として一つの選択肢となる可能性がある．

2）膀胱・尿管子宮内膜症の治療法

膀胱・尿管のいずれにしても泌尿器科との連携が必要である．症状を伴う場合は外科的な治療が優先される．膀胱筋層に浸潤する子宮内膜症は比較的認められるが，尿管の筋層まで浸潤する子宮内膜症はきわめてまれであり，大部分の症例においては尿管周囲を剝離することにより，周囲の内膜症病巣は切除が可能である．しかし，尿管に子宮内膜症が浸潤している症例では，尿管膀胱新吻合や尿管尿管吻合が必要になる．

膀胱表面の子宮内膜症に関しては，骨盤の子宮内膜症の治療法に準じて切除が基本である．尿管口の部分に近い場合は処置が困難なこともあるが，膀胱底部などにある内膜症病変は腹腔鏡下に切除が可能である．尿管口に近い部位の切除，あるいは尿管の切断を伴う形成が必要な場合は尿管カテの挿入など，泌尿器科医と合同で手術的な治療を行うことが必要となる．尿管の閉塞を伴うような症例においては子宮内膜症による閉塞は尿管口に近い症例が多く，尿管膀胱新吻合が必要となる．子宮内膜症の病巣切除と腹腔内の観察は腹腔鏡手術が優れているが，尿管の操作に関してはNezhatや安藤らが報告しているように，かなり熟練した術者でないと腹腔鏡での施行は困難であり[8][9]，開腹手術と腹腔鏡手術との選択は，術者の技量による．

2．小骨盤腔外の子宮内膜症

1）横隔膜・肺の子宮内膜症の治療

治療法に関して現在コンセンサスはないが，胸腔鏡の普及により，繰り返し発症している症例に関しては，外科的診断と治療（切除）が基本となってきている[5]．

2）薬剤による治療法の施行に際して

子宮内膜症の発症した臓器にかかわらず，臨床症状の軽微なものや，手術による切除が困難であったり，治療による合併症が重篤であると判断された場合は薬剤による治療が主体となる．薬剤の種類は表2に示したが，GnRHアゴニスト，ダナゾール，およびピル（低容量，および中容量）が主たる薬剤である．しかし薬剤による治療により，横隔膜子宮内膜症では症状が悪化する症例と寛解した症例の両者が報告されていて，薬物治療施行の可否および有効性について結論は出ていない．また第一選択として薬剤治療を始める場合は，外科的診断をつけずに薬剤による治療を始めることになるので，薬剤による副作用と外科的手術の侵襲を評価し，また妊孕性や挙児希望を考慮しながら，治療法を慎重に決めていく必要がある．

近年では，腹腔鏡と胸腔鏡の普及に伴って，NSAIDsなどの鎮痛剤による経過観察が不可能な症例に対しては，外科的診断と切除による治療が第一選択となってきているのが現状である．

■■ ま と め

異所性子宮内膜症の発症部位は多岐にわたる．その発症機序として転移，化生あるいは胎生期組織遺残の可能性が考えられているが，男性の前立腺周囲においても異所性子宮内膜症が発症することから，化生および胎生期組織遺残が一つの要因であることは明らかである．

一方，左側の骨盤壁や直腸・S状結腸などの月経血が遺残しやすい部位が異所性子宮内膜症の好

発部位であることは，内膜組織の撒布も一つの原因になっていると推測される．異所性子宮内膜症は消化管や呼吸器という機能温存が必要な重要臓器に発症することが比較的多く，産婦人科医としては，ほかの診療科と合同して治療にあたることが望ましい．臨床症状が明らかであるときは外科的治療が優先されているが，薬剤による治療で寛解する症例報告もあり，治療方法は症例ごとに考慮する必要があるであろう．

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〔シンポジウム1／子宮内膜症の病因に関する基礎研究〕

子宮内膜症関連遺伝子同定のための新しいアプローチ

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目 的

月経時に特異的に発現レベルが変動する子宮内膜遺伝子産物は，逆流月経内膜の異所性生着に引き続く子宮内膜症の発症，ならびに，反復する月経周期を通じて内膜症病変が増悪するメカニズムに関与すると考えられる．本研究では，*in vitro* 月経モデルおよびDNAマイクロアレイを用いた網羅的遺伝子解析により，子宮内膜症関連遺伝子を探索し同定することを目的とした．

方 法

患者の同意を得て摘出した良性疾患子宮の内膜より間質細胞を分離・純化した後，17beta-estradiolおよびprogesteroneの非存在下および存在下で2週間培養した．続いて前者は非存在下で（C群），後者は性ステロイド存在下（EP群）と非存在下（withdrawal群，WD群）の2群に分けてさらに2日間培養した．定法により得られた各群のcRNAを約33,000の遺伝子解析が可能なGeneChip®（Affymetrix U133Set）に供し，発現の増強および低下を検討した．C群およびEP群に比較してin vitro月経モデルに相当するWD群において，その発現が2倍以上増強あるいは半分以下に減弱しているものをWD特異的変動遺伝子とした．また，それらの実際の変動をRT-PCRおよびrealtime PCRにて確認した．さらに，各月経周期の内膜組織および手術検体より同意の下に得られた子宮内膜症病変組織よりmRNAを抽出し，RT-PCRで対象とする遺伝子の発現レベルを解析した．

成 績

GeneChip解析で同定されたWD特異的変動遺伝子の中には，月経あるいは内膜症関連遺伝子としてすでに報告されているmonocyte chemotactic protein-1（MCP-1），interleukin-8（IL-8），matrix metalloproteinase-8（MMP-8）が含まれており，本法の戦略と方法の妥当性が示唆された．また，それらは，Gene chip解析と同様，RT-PCRあるいはreal-time PCRによる解析においても同様の挙動を示した．さらに，これらWD特異的変動遺伝子の中で，未知の遺伝子の断片がWD群に強く発現しており，これを一時的にEST-Xと呼称するが，これも同様の挙動をRT-PCRでも確認した．EST-Xは増殖期・分泌期に比べて月経時の正所性内膜に強く発現しているだけでなく，子宮内膜症病変においても，正所性内膜に比べてその発現レベルが上昇していた．

結 論

現在EST-Xの全長遺伝子クローニングを行っているが，本研究で用いた新しいアプローチにより，新規の内膜症関連遺伝子を同定し得る可能性が示された．

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Review Article

The present status of artificial oocyte activation in assisted reproductive technology

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Intracytoplasmic sperm injection (ICSI) is the most effective treatment for achieving fertilization in assisted reproductive technology (ART). However, fertilization failure occurs. The incidence of fertilization failure after ICSI is 1–5%. Approximately 50% of fertilization failure cases could be attributed to the abnormality of sperm factor. As the fertilization fails after ICSI using mature sperm, round spermatids and globozoospermia, artificial oocyte activation may provide a means of improving fertilization rates in such cases. The oocyte activation treatments used in clinical research include calcium (Ca) ionophore treatment, electrostimulation and strontium treatment. In terms of the efficiency of oocyte activation, electrostimulation and Ca ionophore gave better outcomes than strontium treatment. Strontium treatment causes Ca^{2+} oscillations in mice, so it has been viewed favorably. However, in human oocytes calcium oscillation has not been observed. The fertilization rate after ICSI was low in the case of globozoospermia and wiht round spermatids. Some cases of pregnancy were achieved by ICSI alone and oocyte activation methods were not essential in these cases. Among the various oocyte activation methods currently used, it should be noted that issues of genetic safety have not been addressed for the combined use of these oocyte activation methods. (Reprod Med Biol 2008; 7: 133–142)

Key words: calcium ionophore, electrostimulation, fertilization failure, intracytoplasmic sperm injection, oocyte activation.

INTRODUCTION

OOCYTE ACTIVATION IS the process by which oocytes arrested in metaphase II of meiosis are stimulated to resume meiosis.[1] This process is marked by pore formation and secretion in the cortical granules, and release of the second polar body. It is necessary for the initiation of fertilization and occurs when the sperm adheres to the cell membrane of the oocyte. However, it can also be triggered by chemical or physical stimulation in a process known as artificial oocyte activation.

Artificial oocyte activation has been used not only to study the mechanism of oocyte activation,[2] but also in studies of parthenogenesis[3] and nuclear transplantation.[4] Activation of oocytes is precipitated by various types of stimulation, including exposure to ultraviolet light,[5] changes in osmotic pressure,[6] treatment with calcium (Ca) ionophores,[3] electrostimulation,[7] and treatment with strontium,[8] puromycin[9] or cycloheximide.[9]

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Interestingly, the fertilization rate resulting from intracytoplasmic sperm injection (ICSI) is increased when the oocytes undergo artificial oocyte activation in hamsters[10,11] and humans.[12] This raises the possibility that artificial oocyte activation might be used to enhance fertilization rates alongside other techniques. As the fertilization fails after ICSI using mature sperm, immature male germ cells and round head sperm, artificial oocyte activation might provide a means of improving the fertilization rates in such cases.

FERTILIZATION BY INTRACYTOPLASMIC SPERM INJECTION

OOCYTE ACTIVATION OCCURS early in the process of fertilization and involves the resumption of meiosis of oocytes arrested at the metaphase stage of meiosis II. The details of the mechanism are not clear, but a transient increase in calcium ions ($[Ca^{2+}]_i$) inside the oocyte is known to play an important role.[13,14] In ICSI-assisted fertilization, the oocyte is activated by a sperm-derived oocyte activation factor (also known as sperm factor) after the injection.[14–16] A decrease or loss in activity of this factor is a possible cause of fertilization failure with ICSI. When this factor is completely

Table 1 Types of artificial oocyte activation methods and reported cases in clinical research

Types of artificial oocyte activation methods	Mechanism of oocyte activation	Cases of pregnancy and delivery in clinical research
Calcium ionophore†	Increase in Ca^{2+} permeability of cell membrane	+ (Hoshi *et al.* 1995 A23187[17])
Electrostimulation	Pore formation in cell membrane, entry of extracellular Ca^{2+}	+ (Yanagida *et al.* 1999[28])
Strontium	Release of endogenous Ca^{2+}	+ (Yanagida *et al.* 2006[29])
Ethanol	Increase in Ca^{2+} permeability of cell membrane	
Puromycin‡	Inhibition of protein synthesis	+ (Murase *et al.* 2004[30])
Cycloheximide	Inhibition of protein synthesis	

†Calcium ionophores include A23187 and ionomycin. ‡Puromycin is used in combination with A23187.

absent, oocyte activation does not occur and the oocyte remains in metaphase II even if ICSI is carried out. We are able to evaluate the ability of sperm to activate oocytes using mouse oocytes.[17,18] Immobilized sperm injected into the oocyte only undergo decondensation if the sperm nucleoprotein and the oocyte reduction mechanism are normal.[19] When the activity level of the sperm factor is reduced, the oocyte activation mechanism is stimulated at a low level, resulting in a reduction in the level of maturation-promoting factor (MPF) in the oocyte and in the release of the second polar body.[20] However, in some cases, MPF is resynthesized and the activation mechanism of the oocyte is halted. In these cases, premature chromatin condensation (PCC) sometimes occurs after the release of the second polar body and decondensation of the sperm injected into the ooplasm (metaphase III).

Although a transient increase in Ca ions in the oocyte is necessary for oocyte activation, subsequent Ca^{2+} oscillations are not absolutely required. In many oocyte activation methods, the oocyte is activated by inducing a single transient increase in the concentration of Ca^{2+}. Although the functional significance of Ca^{2+} oscillations is not clear, its continuation from the postfertilization to pronuclear fusion stages suggests a role in embryonic development.[21] Furthermore, in mice, parthenogenetic oocytes without Ca^{2+} oscillations develop into blastocysts with a smaller inner cell mass.[22] In addition, Ca^{2+} oscillations affect pronucleus formation,[23] the arrest of early embryo development[24] and post-implantation development.[25]

TYPES AND MECHANISMS OF ARTIFICIAL OOCYTE ACTIVATION

OOCYTE ACTIVATION REQUIRES either a transient increase in Ca^{2+} in the oocyte or a decline in MPF,[26] which normally follows such a Ca spike. Methods of artificial oocyte activation are usually based on one of these mechanisms (Table 1).

Calcium ionophores

Calcium ionophores, including A23187[1] and ionomycin,[31] are commonly used to induce oocyte activation. The first case in which the combined use of A23187 and ICSI resulted in a successful pregnancy and delivery was reported in 1995.[27] This case used the combination treatment to increase the fertilization rate and several subsequent cases have been reported in recent years.[32–36]

Mechanism

Calcium ionophores help activate the oocyte by increasing the Ca^{2+} permeability of the cell membrane, thereby letting extracellular Ca^{2+} flow into the cell.[37] The oocyte activating effect of A23187 is weakened by the presence of protein, including human serum albumin. When a strong effect is desired, a Ca^{2+}/Mg^{+}-free culture medium should be used, and the osmotic pressure should be corrected using polyvinylpyrrolidone. The addition of albumin may be used to stop the ionophore treatment.

For human oocytes, serum-containing medium is preferred for its gentler effect on the oocyte. Following treatment with A23187, the Ca^{2+} concentration in the oocyte peaks after approximately 1 min and then gradually decreases.[37] The treatment causes a single transient increase in Ca ion concentration, but no Ca^{2+} oscillation.

Method

1. Dissolve A23187 (C7522; Sigma, St Louis, MO, USA) in dimethyl sulfoxide (DMSO) to make a 1 mmol/L stock solution. The stock solution should be kept frozen at −80°C.
2. Add 10 μL of the stock solution to 990 μL of culture medium (e.g. human tubal fluid [HTF]) and mix to make 10 μmol/L A23187 treatment solution. Use

the treatment solution as soon as possible after preparation and keep it protected from light exposure until use.
3. Add the A23187 treatment solution to the oocyte and place in a 37°C incubator for 5–15 min.
4. After treatment is completed, immediately wash the oocyte three times with standard culture medium and resume incubation.

When oocyte activation with ICSI alone fails, A23187 treatment may be carried out before or after ICSI to improve the fertilization rates. One report describes treating oocytes with A23187 approximately 30 min after performing ICSI.[27]

Electrostimulation

Electrostimulation is used in studies of parthenogenesis or for embryo cloning in the field of animal science. Fundamental research[11,38] and clinical studies[27] of electrostimulation with ICSI in human oocytes have been reported.

Mechanism

The oocyte is placed between two parallel electrode plates. The electric field generated by a direct current voltage causes charged proteins in the lipid bilayer of the cell membrane to move, thereby forming pores in the membrane.[39] Extracellular Ca^{2+} in the culture medium flow into the oocyte through these pores, transiently elevating the interior Ca^{2+} concentration and activating the oocyte. The lower the electrolyte concentration in the culture medium, the more pore formation is stimulated. The pores are thought to take 10–40 min at 37°C to repair, and longer when the temperature is lower.[40] The concentration of Ca^{2+} is elevated immediately after the application of the stimulus, peaks within 1 min, and then gradually decreases and returns to the original level in approximately 5 min, without subsequent oscillation.[11,37] When electrostimulation was applied repeatedly to imitate Ca^{2+} oscillations in unfertilized rabbit oocytes, parthenogenesis occurred and fetuses were obtained.[41]

Method

The actual process of electrostimulation is described as follows:

1. Fill the electrode chamber with Zimmerman solution[39] or Dulbecco's phosphate buffered saline (D-PBS).[11]
2. Place the oocyte (or multiple oocytes) between the electrodes in the chamber.
3. Apply a rectangular wave of electrostimulation using an electroporator (e.g. apply one pulse of 100–150 V for 50–100 μsec when the distance between the electrodes is 1 mm).
4. Incubate the oocyte in standard culture medium.

The conditions for electrostimulation vary according to the electroporator and chamber type (the distance between the electrodes and the material of the electrodes). Conditions must be set in advance using a 1-day-old oocyte. Although D-PBS is used as the pulsing medium in the above method, 0.3 mol/L mannitol solution (containing 100 μmol/L $CaCl_2$ and 100 μmol/L $MgCl_2$) is more typically used in basic research. Because D-PBS contains more electrolytes, the electric current created by electrostimulation would normally be a problem, but the electric resistance between the electrodes is approximately 13 KΩ or higher, and the electric current created is very weak, at approximately 1 mA. Thus, out of concern that changes in the composition of the culture medium might damage the cell, D-PBS is used.

Strontium treatment method

Because strontium treatment has been shown to cause oocyte activation accompanied with Ca^{2+} oscillations in mouse models,[42] this method is preferred when embryonic development is desired. In contrast, only the initial transient increase in cytoplasmic Ca^{2+} concentration is achieved with A23187 treatment and electrostimulation.[37] Another characteristic of strontium treatment is varied efficacy of oocyte activation depending on the species.[29,43–46] It is most effective in mice, but its efficacy has not been sufficiently confirmed in humans. Nonetheless, cases of pregnancy and delivery have been reported in clinical research applications.[29]

Mechanism

Sr^{2+} induced Ca^{2+} transients in an activated oocyte.[47] However, the mechanism by which Sr^{2+} induces Ca^{2+} oscillations in an oocyte remains unclear. Recently, Ca^{2+} oscillations induced by Sr^{2+} were mediated through inositol trisphosphate receptors.[48] Sr^{2+} is thought to move into the oocyte down the concentration gradient, causing Ca to be released from the endoplasmic reticulum.

Method

1. Dissolve $SrCl_2 \cdot 6H_2O$ (Sigma) in distilled water to make 1 mmol/L Sr^{2+} stock solution and store in the deep freezer.
2. Add 20 μL of the stock solution to 1 mL of a Ca^{2+}-free culture medium (such as HTF) to make 20 mmol/L strontium treatment solution.

3. Place the oocyte into the strontium treatment solution and incubate it for 120 min in an incubator.
4. Transfer the oocyte into standard culture medium and incubate.

The impact of strontium treatment on the chromosomes of gametes has been studied in mice and no effect has been reported.[49]

CLINICAL APPLICATION OF OOCYTE ACTIVATION METHODS

Cases of fertilization failure with ICSI

WHEN ICSI DOES not result in oocyte activation, treatments that artificially activate oocytes are used concomitantly with ICSI to promote fertilization. When ICSI is carried out, the incidence of fertilization failure is 1–5%.[50–54] The number of oocytes used in ICSI is closely associated with the fertilization rate.[52] The incidence of fertilization failure is 13.3% when ICSI is carried out with one oocyte, but decreases to 3.1% with two oocytes and 1% or lower with three or more oocytes. There was no fertilization failure with six or more oocytes.[52] When a small number of oocytes are used in ICSI, the high incidence of failure can be attributed to the poor quality of the collected oocytes or to technical factors (e.g. if a device malfunctioned during the initial ICSI, it would be fixed). Fertilization failure was observed in 5.6% (100/1779) of treatment cycles of ICSI. When fertilization failed in the initial ICSI, the probability of fertilization failure in the second ICSI was 13% and the incidence of such cases is 0.7% in all treatment cycles.[52]

Because the steps between capacitation and sperm–egg fusion are bypassed in ICSI, the problem must lie in later steps of the fertilization process. In particular, abnormalities in oocyte activation, decondensation of the sperm head or pronucleus formation may play a role in fertilization failure. The mechanism of oocyte activation in ICSI is thought to involve the action of phospholipase C zeta (PLCzeta), which is present in the sperm.[53] When the activity of this factor is low or lacking, fertilization fails. In addition, after fertilization, the sperm needs to undergo decondensation, which depends on the condition of the sperm nucleoprotein as well as the reduction system in the oocyte.[19] For example, decondensation can be impaired when the zinc supply from the seminal plasma is decreased because of a disorder, such as prostatitis, or when a large number of disulfide bonds are formed in the nucleoproteins.[55] The sperm aster also plays an important role in the fusion of male and female pronuclei. Abnormalities in the sperm centrosome that comprise the sperm aster can impair decondensation.[57] When oocytes that were not successfully fertilized following ICSI were observed using chromatin staining, oocyte activation failed to occur in 70% of the oocytes, even though the sperm was successfully inserted into the oocyte.[52] Moreover, when ICSI was carried out using sperm from volunteers whose sperm had been shown to be capable of fertilizing oocytes, fertilization was successful in 70% of the cases. Therefore, for approximately 50% of the oocytes that were not fertilized following ICSI, the failure of fertilization could be attributed to the sperm.[52]

If abnormalities in oocyte activation factor activity are the cause of fertilization failure, the combined use of oocyte activation treatments and ICSI can be considered. However, if the source of the problem lies elsewhere, there is currently no treatment method. The recurrence rate of fertilization failure following ICSI is 13%, which means that in many cases, fertilized oocytes can be obtained from a second round of ICSI.[52] When the risk of fertilization failure is high, however, an oocyte activation treatment combined with ICSI has been used. The oocyte activation treatments used in clinical research include Ca ionophore treatment, electrostimulation and strontium treatment. Table 2 shows the results of clinical research on the combined use of oocyte activation and ICSI in patients with fertilization failure and low fertilization cases following an ICSI attempt, excluding cases using spermatid and those with globozoospermia. We reported the first case of pregnancy and delivery after ICSI with electrostimulation. Since then there have been 10 delivery cases using the same treatment as the fertilization failure case. In terms of the efficiency of oocyte activation, electrostimulation and A23187 gave better outcomes than strontium treatment. Strontium treatment showed variable efficacy. This treatment was developed in studies using mice and has been viewed favorably because it causes Ca^{2+} oscillations and is similar to the physiological stimulus that activates oocytes.[41] However, in human oocytes, Ca^{2+} oscillations have not been observed following treatment and its role in human oocyte activation is not clear.[29] For clinical research, methods with known mechanisms should be selected. Chromosomal analysis of oocytes fertilized using both ICSI and oocyte activation did not show an increased risk from electrostimulation[56] and A23187 + puromycin.[50]

The timing of electrostimulation relative to ICSI is another important factor to consider. When electrostimulation is carried out prior to ICSI, the oocyte tends to

Table 2 Reports on the combined use of oocyte activation methods and intracytoplasmic sperm injection†

Reference	Indication	Types of oocyte activation methods	Cases of pregnancy and delivery
Tesarik *et al.* (1994)[32]	Fresh oocytes	A23187	Research‡
Hoshi *et al.* (1995)[27]	Low fertilization	A23187	Two cases of pregnancy
Yanagida *et al.* (1999)[28]	Fertilization failure	Electrical stimulation	One case of pregnancy and delivery
Yamano *et al.* (2000)[57]	1-day-old unfertilized oocytes	A23187 + puromycin	Research‡
Nakagawa *et al.* (2001)[58]	1-day-old unfertilized oocytes	A23187 + puromycin	Research‡
Tesarik *et al.* (2002)[59]	ICSI patients	Mechanical	One case of pregnancy and delivery
Eldar-Geva *et al.* (2003)[52]	Fertilization failure	A23187	One case of pregnancy and delivery
Murase *et al.* (2004)[30]	Fertilization failure	A23187 + puromycin	One case of pregnancy and delivery
Ebner *et al.* (2004)[60]	Fertilization failure	Mechanical	One case of pregnancy and delivery
Chi *et al.* (2004)[31]	Low fertilization	A23187	One case of pregnancy and delivery
Heindryckx *et al.* (2005)[34]	Fertilization failure	A23187	Three cases of pregnancy
Lu *et al.* (2006)[61]	1-day-old unfertilized oocytes	A23187 + puromycin	Research‡
Manipalviratn *et al.* (2006)[62]	1-day-old unfertilized oocytes	Electrostimulation	Research‡
Ahmady *et al.* (2007)[35]	Immotile testicular sperm	A23187	One case of pregnancy and delivery
Moaz *et al.* (2006)[63]	Low fertilization and fertilization failure	Ionomycin	Research‡
Yanagida *et al.* (2006)[29]	Low fertilization	Strontium	One case of pregnancy and delivery
Nasr-Esfahani *et al.* (2008)[36]	Low fertilization (teratozoospermia)	Ionomycin	Two cases of pregnancy

†Excluding cases using spermatids and globozoospermia. ‡'Research' means that this report was carried out for the purpose of research not a therapeutic purpose. ICSI, intracytoplasmic sperm injection.

degenerate. It is possible that the cell membrane is not sufficiently repaired after electrostimulation[40] and is further damaged by ICSI. Because the cell membrane can also be damaged by Ca ionophore treatment, we treat oocytes with Ca ionophore 30 min after ICSI. In addition, in mice, when the sperm is present during electrostimulation, the incidence of chromosomal aberration (structural abnormality) in the sperm is approximately 50% higher. In such cases, the chromosomes of the oocyte were unaffected. In contrast, when electrostimulation was carried out 30–60 min before ICSI, there were no chromosomal abnormalities, but the oocyte still tends to be damaged.

Special cases of ICSI include cases of round-headed spermatozoa (globozoospermia) and the use of spermatids. Round-headed spermatozoa possess round heads lacking an acrosome.[65] In spermatozoa, the level of sperm factor appears to be low and there are reported cases of fertilization failure associated with it, for which clinical application of ICSI has been reported (Table 3). The reported fertilization rates were 0–42% with ICSI alone and approximately 70% in conjunction with oocyte activation methods. With round-headed spermatozoa, many cases of pregnancy were achieved by ICSI alone[66,68,71–74,77] and oocyte activation methods were not essential. From clinical research using round spermatids, 14 cases of pregnancy have been reported to date (Table 4).[78,79,81,82,84–86,93,94] Because the activity level of the sperm factor is low in human round spermatids, oocyte activation in conjunction with ICSI has been considered. However, the reported fertilization rates were between 16 and 69% with ICSI alone.[78,79,81–89,92,95,96] Oocyte activation methods were used along with ICSI in a small number of reported cases and the fertilization rate was approximately 40%.[80,81] In our experience, the fertilization rate with round spermatids was 17%.[97] Elongated spermatids seem to have a similar oocyte activation capacity as mature sperm.

ASSISTED ACTIVATION

ASSISTED ACTIVATION IS based on the idea of using oocyte activation methods in conjunction with ICSI in patients without fertilization failure. These patients do not exhibit a low fertilization rate, but this approach is used to further raise the fertilization rate and increase the number of embryos obtained. As this method works on the oocytes that are still unfertilized after ICSI, the number of fertilized oocytes should increase. However, fertilized oocytes would also undergo

Table 3 Reported cases of intracytoplasmic sperm injection for globozoospermia

Reference	Oocyte activation treatment	Fertilization rate (%)	Cases of pregnancy and delivery
Liu *et al.* (1995)[66]	None	–	Two cases of pregnancy
Trokoudes *et al.* (1995)[67]	None	50	One case of pregnancy
Bourne *et al.* (1995)[68]	None	–	None
Battaglia *et al.* (1997)[69]	None	10	None
	A23187	75	None
Rybouchkin *et al.* (1997)[70]	A23187	–	One case of pregnancy
Stone *et al.* (2000)[71]	None	10–42	One case of pregnancy and delivery
Kim *et al.* (2001)[72]	A23187	60	One case of pregnancy and delivery
Zeyneloglu *et al.* (2002)[73]	None	31	One case of pregnancy and delivery
Nardo *et al.* (2002)[74]	None	–	One case of pregnancy and delivery
Kilani *et al.* (2004)[75]	None	38	One case of pregnancy and delivery
Heindryckx *et al.* (2005)[34]	A23187	71–77	Five cases of pregnancy
Khalili *et al.* (2007)[76]	None	0 (four cases)	None
Dirican *et al.* (2007)[77]	None	9, 33 (two cases)	Two cases of pregnancy and delivery

Table 4 Reports on round spermatid injection

Reference	Oocyte activation treatment	Fertilization rate (%)	Cases of pregnancy and delivery
Tesarik *et al.* (1995,96)[78,79]	None	36	Two cases of pregnancy and delivery
Tanaka *et al.* (1996)[80]	Electrostimulation	42	One case of pregnancy
Vanderzwalmen *et al.* (1997)[81]	None	16	None
	A23187	36	One case of pregnancy
Antinori *et al.* (1997)[82]	None	56	Two cases of pregnancy
Yamanaka *et al.* (1997)[83]	None	69	None
Amer *et al.* (1997)[84]	None	25	Four cases of pregnancy
Kahraman *et al.* (1998)[85]	None	26	One case of pregnancy
Barak *et al.* (1998)[86]	None	27	One case of pregnancy and delivery
Al-Hasani *et al.* (1999)[87]	None	18	None
Ghazzawi *et al.* (1999)[88]	None	22	None
Levran *et al.* (2000)[89]	None	44.9	None
Vicdan *et al.* (2001)[90]	None	–	None (0/6)
Khalili *et al.* (2002)[91]	None	–	None (0/7)
Sousa *et al.* (2002)[92]	None	16	None (0/33)
Saremi *et al.* (2002)[93]	None	–	One case of pregnancy and delivery
Amarin *et al.* (2002)[94]	None	–	One case of pregnancy
Ulug *et al.* (2003)[95]	None	42	None
Benkhalifa *et al.* (2004)[96]	None	36	None

treatment. Because the techniques of oocyte activation are still at the clinical research stage, they should not be used for such a purpose. When two or fewer oocytes are used in ICSI, the risk of fertilization failure is higher. When oocyte activation methods were concomitantly used with ICSI in such cases, the fertilization rate, embryonic development rate and pregnancy rate did not show any significant difference and, therefore, these methods were evaluated to be ineffective.[32]

RESCUE ACTIVATION

IN RESCUE ACTIVATION, oocyte activation is used to promote fertilization in oocytes that remain unfertilized following ICSI when the fertilization status is evaluated. The time interval between the collection of the oocyte and the oocyte activation treatment is important. Ontogenesis cannot be expected after aging of the oocyte. With 1-day-old oocytes, the pregnancy rate is extremely

low. When rescue activation was carried out for such oocytes in 52 cases, the fertilization rate was 78% and two cases of pregnancy were obtained, but both resulted in miscarriage.[52] Therefore, this method should not be used clinically for 1-day-old oocytes.[98,99] Rescue ICSI 6 h after insemination has already been shown to be effective in cases of fertilization failure with *in vitro* fertilization.[100] In the same way, rescue activation should be carried out within 6 h after ICSI. When oocyte activation does not occur following ICSI, the head of the injected sperm may undergo decondensation and PCC may occur over time. Premature chromatin condensation is the abnormal condensation of chromatin and can induce damage in the chromosome or DNA. Therefore, an evaluation of fertilization should be done before PCC takes place.[101] Because the incidence of PCC is elevated starting 4 h later when oocyte activation does not occur following ICSI, it is recommended that the fertilization status be evaluated and oocyte activation treatment carried out on unfertilized oocytes within 4 h.[102] If at least one fertilized oocyte is found at the evaluation, oocyte activation treatment is not necessary.

CONCLUSION

ARTIFICIAL OOCYTE ACTIVATION is frequently used in basic research in the field of reproductive technology. Oocyte activation occurs at the initial stage of fertilization and is essential for fertilization. In some cases of fertilization failure with ART, oocyte activation did not occur because of abnormalities in the sperm-derived oocyte activation factor. In 50% of the unfertilized oocytes following ICSI, the failure can be attributed to problems with the sperm.[52] When fertilization failure is predicted with ICSI, oocyte activation methods can be used along with ICSI in a clinical research setting. Oocyte activation methods can also be used as a rescue procedure when fertilization failure is detected within 4 h of ICSI.[101] Among the various oocyte activation methods currently used, Ca ionophore treatment is the easiest to use. However, it should be noted that issues of genetic safety and abnormal imprinting have not been addressed for the combined use of these oocyte activation methods.[102]

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顕微授精

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はじめに

卵細胞質内精子注入法（intracytoplasmic sperm injection：ICSI）は体外受精で受精をはかることができない場合に適応となる媒精法の一法である．1992年にベルギーのグループがその成功を報じて以来，多くの重症男性因子例や受精障害例へ用いられてきた．安定した高い受精率が得られること，多精子受精の心配がないことが長所で，卵子損傷による変性が起こりうること，体外での操作による遺伝的影響の実態が不明なことが短所である．また，当面の課題としては，卵子と精子が存在するのに受精率が100％にならないことへの解決法を考案すること，良質な胚を作成するための良質な配偶子を選別する方法を考案することが挙げられる．以上のことをふまえて，ICSIの実践に役立つ重要なポイントを解説する．

マイクロマニピュレーション機器のセットアップ

詳細は成書に譲り，重要なポイントを述べる．マイクロインジェクターとマニピュレータのセットアップを行う．また，ICSIのための注入用ニードル（マイクロピペット）と卵保持用のホールディングピペットも取り付ける．機器をどのように調整するかは術者の好みが加わるところであるが，主役は配偶子ということを忘れてはならない．術者に都合がよいセットアップがベストではなく，卵子や精子にやさしいセットアップがベストである．例えば，インジェクターとチューブには，シリコンオイル，（古くは）フロリナート，蒸留水，空気などが充填されている．このなかで，空気を用いた場合ではそのメンテナンスが容易であるが，ニードルによるインジェクション操作（精子の吸引・排出など）は当然レスポンスが悪いので，トレーニングである程度は克服できると思うが，思わず大量の細胞質を吸引したり，大量の培養液を注入してしまうトラブルが発生しやすい．これらの操作上の問題はトレーニングにより解決できるかもしれないが，配偶子に傷害を与えるリスクをできる限り排除することを第一にするのであれば，多少面倒でもオイルや蒸留水をチューブングの充填剤として使うことが望ましいと思われる．

ICSIを実施した卵子の数ごとの受精障害発現率（すべての卵子が受精しなかった場合の出現頻度）を調べると，卵子数が少ないほど高く，卵子数が1個では20～25％が受精障害となることが判明している[1]．この原因として，セットアップ後，初めてのICSI操作ではセットアップのコンディションが十分でないことが考えられる．これからのARTでは採取する卵子数が減少するので，機器のコンディションを常にベストに保つことが要求される．

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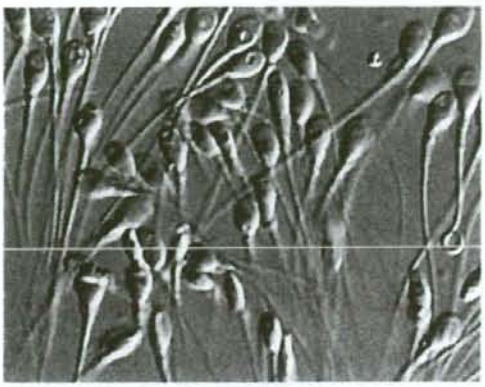

図1　精子頭部の空胞

OlympusIX71，高倍率精子観察システム（対物レンズにドライタイプ×60使用，最終拡大倍率6,600倍）．CCDカメラは汎用型の低解像度機．頭部に大小の空胞が観察される．

精子の選択

奇形精子症のICSIでは妊娠率が低下するように，精子の質が初期胚の発生や着床に関与するので，ICSIを行うには良質の精子を選択する必要がある．通常は運動性が良好な形態正常な精子を選択して不動化処理を行う．運動性が良好な精子では精子DNAやミトコンドリアDNAの断片化が少ないと報告されている[2,3]．受精率などへの影響が観察されているが，着床率などについては有意差が認められていない．

2002年ごろからノマルスキー微分干渉装置とビデオカメラシステムを用いて運動精子を6,000倍に拡大して形態を観察し，良好形態精子を選別する試みがBartoovら[4,5]によって行われてきた．彼らの最近の報告によれば，正常形態の精子でも頭部に0.78±0.18 μmを超える空胞（vacuole）を認めた場合では，妊娠率が低く（18％vs 50％），流産率が高い（80％vs 7％）ことが判明した[6]（図1）．この場合，受精率や良好胚率などについては有意差を認めていない．Hazoutら[7]も，38歳未満で2回以上のICSIで妊娠が得られなかった症例に対して，正常形態で頭部に空胞がない精子を選択してICSIを行った報告をした．それによれば，Berkovitzらと同様に，受精率，良好胚率には有意差を認めなかったが，着床率，生産率は有意に高かった．さらに興味深いのは，精子DNA断片化率が高い症例（＞40％）でも，この基準で精子を選別すると高い着床率（33.3％），生産率（28.6％）が得られたことである[7]．つまり，頭部に空胞がない精子はDNA断片化が少ないと考えられる間接的根拠が示されたことになり，形態による精子選別の重要性が示唆されている．

精子の不動化

精子の不動化処理の目的は精子の動きを止めて精子をピックアップしやすくすることではない．精子を卵子内に送り込んで（注入して），受精を開始させる必要がある．受精の開始には以下の2項目が必要である．

（1）卵子が第二減数分裂中期で停止していた減数分裂を再開させること．

（2）硬くコンパクトにパッキングされている精子のクロマチンをDNA複製ができるように緩めること．

前者については精子が精子頭部に持っている卵活性化因子（精子型フォスフォリパーゼCゼータ，PLCζ）が卵子内に拡散する必要がある．そのためには，卵子内に注入された精子頭部のバリアとなっている細胞質膜を除去する必要がある．細胞膜を除去する方法としては，Triton-X処理，ソニケーションなどで簡単に行えるが，精子DNAに影響を及ぼさないで細胞膜を除去する方法となると適当なものがないのが現状である．そこで，物理的に精子細胞膜に修復できない大きな傷をつけて，細胞全周にわたって細胞膜を破壊する方法が考案された．精子頭部の細胞膜を損傷するのが効率がよいが，頭部へのダメージを避けて精子尾部に損傷を与えるのが「精子不動化処理」である．したがって，処理としては精子頭部に近いほど，処理が強いほどよい．

注入用ピペットの先端で精子尾部（中片部に近い場所）をしごいて不動化処理を行うが，精子尾部がちぎれる程度に行ってもよい．尾部が切断された場合には，その精子を使用せず，別の精子を不動化し直す．卵子内に取り込まれた尾部の役割についてはよくわかっていない．ハムスターでは

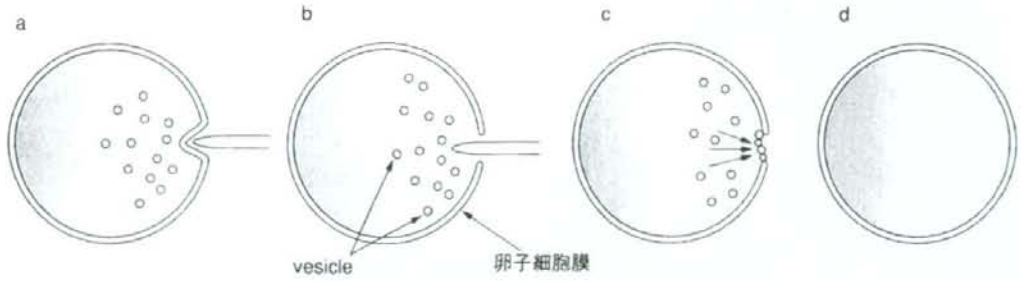

図2　修復機序1

細胞膜に穴が形成されたときの修復過程[9]．a：細胞膜にニードルが接触．b：ニードルが当たると細胞膜はすぐに切れてしまい貫通した．c：細胞外液とCa^{2+}濃度勾配により，細胞内に存在したvesicleが穴のところに瞬時に集合する．d：集合したvesicleは互いに癒合し，穴をシールし修復する．

8細胞期胚までは尾部のコンポーネントが各割球に認められていることがわかっている．実施上，注意が必要なことは，注入用ピペット先端を曲げたもの（30度など）を使用する場合，セッティングが甘いと（先端が浮き気味になっていると），不動化が十分に行われないことがある．不動化処理の甘さは，受精時のカルシウムオシレーション発現遅延[8]，場合によってはそのために精子DNA損傷（精子クロマチンがpremature chromosome condensationを起こすため）を起こすことも考えられる．受精開始の遅延は，初期胚発生の異常を起こすことがある．

後者については，精子が卵子内に注入されたのちは速やかに脱凝縮過程に入る必要がある．そのためには，やはり細胞膜がバリヤとなるので，しっかりとした「不動化処理」が必要である．細胞膜の崩壊につれて，卵細胞内の還元システム（還元型グルタチオンなど）により，ジスルフィド結合（S-S）がチオール基（-SH）となり，核蛋白のプロタミンがヒストンに置換され，脱凝縮が進む．

1．精子不動化を行う培養液

精子の不動化を行う培養液はヘペス緩衝培養液である．この培養液にpolyvinyle pyrrolidone（PVP）を添加して（最終濃度6～8%）用いることもある．PVPは古くは代用血漿として用いられていた経緯があり，また現在では錠剤の被覆や化粧品にも多用されていて，その安全性については比較的安全なものと思われる．しかし，組織や人体に安全であっても1個の細胞である卵子に対して安全とはいい切れない．もとより非生理的物質であるので，卵子内に注入しないことに越したことはない．PVPの効用としては，マイクロニードルの管壁に異物が付着するのを防ぐこと，その粘性から運動精子のピックアップがしやすくなることが挙げられる．PVPを使用しないでICSIを実施できればよいが，使用したほうがより安全に確実にICSIを実施できるのであれば，PVPを使用してよいと思われる．現在のところ，PVPを使用したほうがICSIの成績が不良になるという報告はない．

ICSIニードルの穿刺

ICSIニードルの穿刺および精子の注入では，卵細胞が変性しないように穿刺すること，確実に卵細胞内に精子を注入すること，精子卵子相互作用が生じやすい状態にすることなどが重要なポイントである．もちろん第一極体を認める反対側の半球に穿刺する．

1．卵子生存率を上げるポイント

細胞膜は脂質二重層と呼ばれる構造をとる．穿刺で細胞膜が損傷した場合の修復に関する研究によれば，開孔した部位に隣接する外側に，卵細胞質内に存在する"vesicle（脂質二重層からなる小塊）"が瞬時に移動して配列する．その後にvesicle-vesicle fusionを起こして脂質二重層が修復される[9]．このような修復が行われるのは，ニー

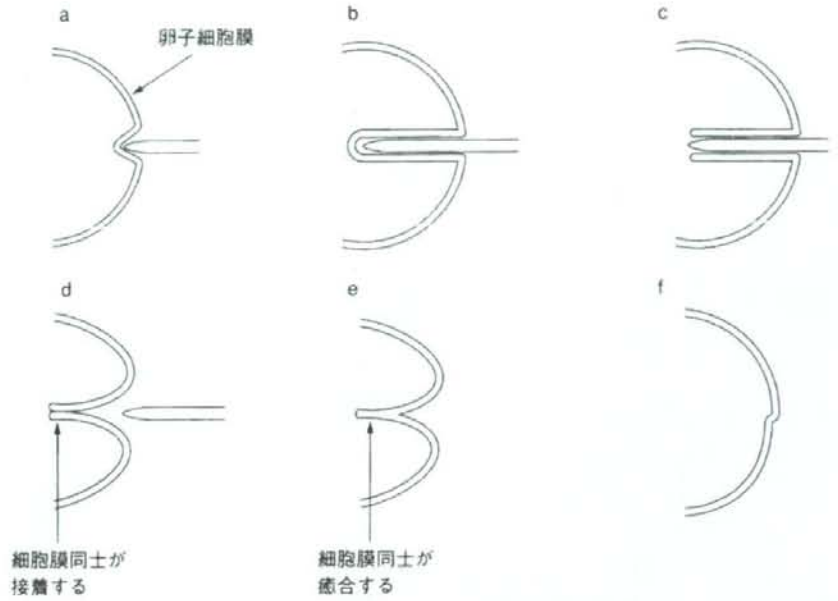

図3　修復機序2

ニードルを刺してもすぐに細胞膜が切れないで，かなり刺してから切れる場合の修復過程．a，b：ニードルを卵子に穿刺すると，なかなか穿刺できず，細胞膜が嵌入する．c：細胞膜が十分に引き延ばされてからやっと細胞膜が切れた．d：精子を注入後，ニードルを静かに引き抜くと，引き延ばされていた細胞膜が互いにすぐに接着する．e：接着した細胞膜が癒合する．f：細胞膜の修復が完了する．

ドルを刺入した場合に，刺入と同時に細胞膜が破れて小孔が開口してしまう場合である（図2）．もう1つの修復機序はニードルを刺入していってもなかなか貫通できない場合で，ニードルを刺入するにつれて細胞膜が引き込まれていく．そして卵細胞質内にかなり引き込まれてから細胞膜を貫通する．精子を注入後にニードルをゆっくりと抜去すると，引き延ばされた細胞膜同士が互いに接着するので，ニードルを抜去すると直ちに細胞質の修復が行われる（図3）．細胞膜の修復に関しては，こちらの修復のほうが優れているといえる．

ICSIでの生存率，受精率が高いピエゾICSIは後者の修復機序を狙った方法である．ピエゾICSIに用いられるニードルの先端は平坦（ストロー状）で鋭利ではない．当然，穿刺によって細胞膜に開く穴は，先端が鋭利な通常のニードルに比べるとはるかに大きい．しかし，卵子の生存率はピエゾICSIのほうがはるかに高い．これは，ニードルを卵子に刺入するときに，先端が鋭利でないがゆえに，なかなか穿刺できずに卵細胞膜が十分に引き延ばされ，そこで細胞膜を穿刺するからである．

精子の注入

卵細胞質内にニードルを刺入したら精子を注入するが，このときには精子とともに注入される精子浮遊液の量が最少になるように調整する．液量が多いとICSIの成績が明らかに低下するというデータはないが，理論的に注入される液は生理的でないこと，液が精子・卵子インターアクションを障害する可能性があることがその理由である．

ICSIニードルの抜去

精子注入後はニードルを穿刺した傷口が最小になるように静かに抜去する．

おわりに

ICSIで最も重要なことは精子を確実に安全に注入することである．そのためには，適切な外径の先端が鋭利な注入用ニードルで卵子を穿刺するが，なるべくニードルが卵細胞膜を穿刺しないように，ニードルを静かに進めて穿刺し，精子だけを注入することである．さらに重要なことは，精子不動化処理を確実にしっかりと行うことである．

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特集　生殖医療の現状と問題

体外受精・顕微授精

柳田　薫*

キーワード　生殖補助医療　体外受精　顕微授精

はじめに

現在の不妊治療の柱は，タイミング法と生殖補助医療（assisted reproductive technology；ART）である．生殖補助医療といえば通常は体外受精・胚移植（*in vitro* fertilization and embryo transfer；IVF-ET，IVF）と顕微授精を指すが，広義には配偶者間人工授精（artificial insemination with husband's semen；AIH）も含まれる．

顕微授精には数種類の方法があるが，現在では完成度が最も高い卵細胞質内精子注入法（intracytoplasmic sperm injection；ICSI）が選択されて実施されている．IVFはSteptoeとEdwardsにより1978年に[1]，ICSIはPalermoらによって1992年にその成功（妊娠・出産例）が報告された[2]．IVFの最初の成功例は両側卵管摘除による卵管因子例で，それまでの治療法では絶対に妊娠が不可能な症例であった．その後，IVFは表1のような場合に適応とされ実施されている．そして，ICSIはIVFを行っても受精しない場合に適応とされる（表1）．したがって，現代の種々の不妊治療のなかで，IVFやICSIといったARTが重要な役割を担っていることは容易に理解できる．本稿では，不妊治療におけるARTの位置付け，成績，副作用と遺伝的安全性について述べる．

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表1　体外受精，顕微授精の適応

IVFの適応
1. 卵管性不妊症
2. 男性因子
3. 免疫性不妊症
4. 原因不明不妊症

ICSIの適応
1. 乏精子症，精子無力症
2. IVFの受精障害
3. 精巣精子回収法を実施した場合

Ⅰ．不妊治療のなかのARTの位置付け

不妊治療の基本的な柱はタイミング法，人工授精，体外受精の3本である．顕微授精は，体外受精で受精障害となる場合の媒精法（受精を図る方法）のオプションと考えられる．

不妊症の原因は多岐にわたるが，それぞれの不妊原因での基本的な治療法選択の流れを図1に示した．不妊原因には卵管性，免疫性，子宮性，排卵性，原因不明そして男性因子を挙げたが，実際の症例では複数の原因をもつ場合が多々あるので考え方がより複雑になる．

女性側原因に対する治療法はタイミング法，人工授精，体外受精の順に選択され進行する．原因によっては，たとえば卵管因子ではまず，可能な限り卵管形成術などのような手術療法を考えるが，卵管のダメージの状況によっては，最初から体外受精を選択する場合もありうる．排卵障害例では，その排卵障害を排卵誘発剤で治療し，改善したうえでタイミング法を実施する．しかし，それでも妊娠に至らない場合には

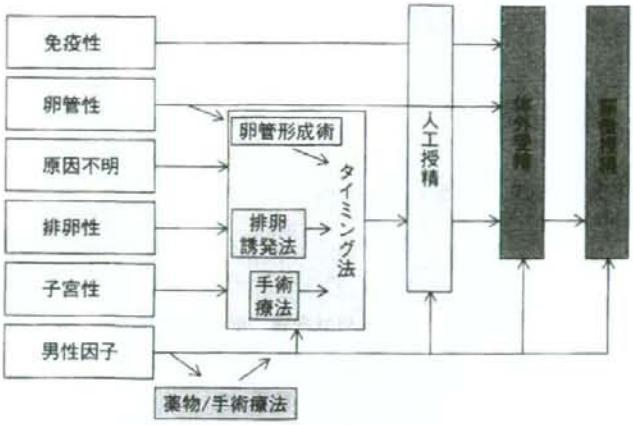

図1　不妊治療の流れ

表2　治療法別出生児数および累積出生児数（2005年）

	治療周期総数	出生児数	累積出生児数
新鮮胚(卵)を用いた治療	42,822	6,706	75,711
凍結胚(卵)を用いた治療*	35,069	6,542	30,194
顕微授精を用いた治療	47,579	5,864	48,964
合計	125,470	19,112	154,869

*凍結融解胚を用いた治療成績と凍結融解未受精卵を用いた治療成績の合計
（平成18年度日本産科婦人科学会倫理委員会・登録・調査小委員会報告．日産婦会誌 2007；59：1717-1739より引用）

人工授精が選択される．そして，人工授精でも妊娠に至らなければ，体外受精が治療の候補となる．

一方，男性因子は精子濃度や運動率からの評価によりタイミング法，あるいは人工授精，体外受精そして顕微授精がダイレクトに選択される．そして，選択された治療法で妊娠に至らなかった場合には，治療がステップアップされる．

2005年にはIVFやICSI（凍結胚移植例も含む）で誕生した児は1万9,112名と登録されている（**表2**）[3]．この年の出生数が106万7,000名であるので，出生した児の1.8%がART由来の児となり，子ども56名に1名の割合である．この全出生児に対するART児の占める割合が年々増加していることは周知の事実である．ARTを実施している施設においては妊娠の38.6%がARTによるものである（厚生科学研究，1999年）．不妊センターを開設して3年目の自施設でも妊娠例の33.3%がARTによる妊娠である．これらのことから，ARTは不妊治療にはなくてはならない重要な治療法であると認識できる．

Ⅱ．ARTの成績

本邦でIVFやICSIを実施するためには，日本産科婦人科学会へ実施施設と実施者の登録，結果の報告を行う義務があり，学会の倫理委員会がARTの結果を年報として公表している．最新の報告は平成17年分の成績で，分かりやすく改変し，**表3**に示した[3]．

IVFは4万2,685周期に計画され，実際には4万334周期で採卵が行われた．採卵当たりの妊娠率が22.0%，移植当たりの生産率が22.9%となっている．一方，ICSIでは4万4,553周期で治療が計画され，4万2,478周期で採卵が行われた．ICSIがIVFの件数を上回っている．採卵当たりの妊娠率が17.7%，移植当たりの生産率が19.0%で，IVFよりもやや下回っている成績である．ICSIでも射出精子以外の精子を用いた場合（精巣上体や精巣から回収された精子）では，2,689周期で採卵され，採卵当たり妊娠率は16.9%，移植当たりの生産率は17.8%であり，射出精子を用いた場合と同等の成績を示した．

表3に示した妊娠率は1治療周期の妊娠率である．複数回のARTを行い妊娠に至る場合もあり，ART実施回数ごとの累積妊娠率を求めると，妊娠例の90%が含まれる実施回数は5回となる[4]．また，最終的に妊娠できるのは治療を開始した症例の約50%であり，残りの50%はARTでも妊娠が困難である．