In vitro fertilization by intracytoplasmic sperm injection

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Summary

Intracytoplasmic sperm injection (ICSI) is the latest, and by far the most efficient, variant of micromanipulation-assisted fertilization, whereby a single spermatozoon is selected, aspirated into a microinjection needle and injected to the oocyte cytoplasm. The development of this technique is mainly linked to application in human assisted reproduction for which it enables fertilization with defective spermatozoa that would not otherwise be able to penetrate an oocyte by their proper means. Because ICSI by-passes many steps of the natural fertilization process, it offers an extremely interesting model for the study of basic mechanisms underlying fertilization. This is particularly true for oocyte activation, whose mechanism needs to be revisited in light of the current ICSI research. The massive application of ICSI in human infertility treatment also represents a huge laboratory in which the impact of different genetic and epigenetic anomalies of the male gamete on fertilization and embryonic development can be studied. *BioEssays* 21:791–801, 1999.

Introduction

Sexual reproduction is mediated by specialized cells, the male (spermatozoon) and the female (oocyte) gametes, whose chromosome and DNA content has been reduced to half (haploid) compared with diploid somatic cells. This reduction is mediated by two successive meiotic divisions, of which only the first is preceded by a DNA synthetic phase, and is realized during gametogenesis (spermatogenesis and oogenesis) within the testis and the ovary. During fertlisation, spermatozoon and an oocyte unite to create a diploid zygote, whose future successive mitotic divisions give rise to all cells of a new individual. In most invertebrates and nonmammalian vertebrates, adult animals deposit gametes outside their body, where fertilization subsequently takes place. In contrast in mammals, fertilization occurs within the female genital tract. Hence, in vitro fertilization (IVF) and early embryonic development can be achieved relatively easily in invertebrates and nonmammalian vertebrates with extracorporeal mode of natural fertilization, whereas re-creation in vitro of the local environment of the female genital tract, required for mammalian IVF, is a much more difficult task.(1)

The efficacy of mammalian IVF remained low until the late 1950s, when substantial improvements were made mainly due to two important innovations, the development of in vitro culture techniques that allowed spermatozoa to undergo preparatory processes rendering them capable of fertilization (sperm capacitation) and the use of hormonal stimulation regimens to increase the number of mature oocytes recovered from the ovaries (superovulation). The first successful application of IVF in the treatment of human infertility occurred in the late 1970s(2) and, in the following years, assisted human reproduction soon became the main application of mammalian IVF. With the increasing number of human IVF attempts, indications of IVF treatment, which originally concerned only female infertility due to obstruction of oviducts, was extended to include moderate forms of male infertility. However, at least several tens of thousands of spermatozoa with adequate motility, morphology, and capacity to respond to physiologic stimuli were still required for successful IVF.

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Because most IVF failures were associated with inability of the sperm to penetrate the zona pellucida, a rigid glycoprotein coat surrounding the oocyte (Fig. 1), it became evident that further improvements of IVF efficacy in cases of sperm insufficiency might be possible by assisting this critical step of the human fertilization process. The first report on fertilization of mammalian (hamster) oocytes by intracytoplasmic sperm injection[3] (ICSI) demonstrated the feasibility of this approach. Substantial improvement in cell micromanipulation techniques in the 1980s raised the possibility that similar techniques might also be used in humans. After initial trials, during which less invasive approaches to assist penetration of the zona pellucida were attempted, ICSI (Fig. 1) proved the most efficient method. Pioneered by Lanzendorf et al.,[4] who curiously failed to capitalize on this process, human ICSI was perfected and introduced extensively into the treatment of human male infertility by a group of investigators working at the Dutch-speaking Free University of Brussels.[5,6] Under insistent pressure by infertile couples, who saw in ICSI their last chance to procreate, clinical application of this method spread rapidly throughout the world. Knowledge obtained through this human application has contributed significantly to the understanding of the mechanisms that govern mammalian oocyte activation, whereas experimental studies of animal ICSI have lagged behind because of species-specific difficulties encountered in the mouse, the species most commonly used in experimental studies of fertilization (see below). Despite this, the ICSI technique has now been adapted to a number of mammalian species, and many problems raised by human application are being addressed in animal models.

This review begins with an overview of general reproductive biology issues relevant to the use of ICSI for fertilization before discussing special problems of human ICSI in which the impact of the pathologic process underlying male infertility plays a central role. Current topics of ICSI research, including new applications in human assisted reproduction, are also mentioned.

By-passing steps of the fertilization process

Outline of the main biological events underlying fertilization

Oocytes are large cells that contain the female haploid genome along with all necessary elements to support the early development of the future embryo. The cell cycle of mammalian oocytes is blocked in a final phase of meiosis until fertilization. Spermatozoa, in their turn, are small motile cells whose role is to bring the male haploid genome into the oocyte and to generate signals that enable the oocyte to overcome the cell cycle block, to exit meiosis, and to enter the mitotic embryonic cell cycle. These functions are mediated by two cell-activation events that occur sequentially in the spermatozoon and in the oocyte. Sperm activation is stimulated by components of the oocyte cell coat, the cumulus oophorus and the zona pellucida, and this process involves the selection of the most responsive spermatozoon from the large number of less-responsive candidates. In turn, the responsiveness of sperm to stimulation by components of the oocyte cell coat develops during exposure of spermatozoa to the female genital tract (sperm capacitation), and this process can be mimicked by sperm incubation in appropriate culture media.

As a result of activation, spermatozoa undergo the acrosome reaction in which they shed the contents of the acrosome localized in the anterior portion of the sperm head, and are able to penetrate the zona pellucida and fuse with the oocyte. Sperm-oocyte fusion triggers oocyte activation, which leads to modifications of the zona pellucida that prevent less advanced spermatozoa from penetration (block to polyspermy) and starts the cell cycle events necessary for completion of meiosis and the beginning of embryonic development.

Sperm capacitation and acrosome reaction: a species-dependent issue

Changes that occur during sperm capacitation are beyond the scope of this review. From the biological point of view, however, these changes are necessary for the development of a particular pattern of movement, known as sperm hyperactivation, and of the ability to undergo the acrosome reaction in response to components of the oocyte cell coat. Both of these events are crucial for active sperm penetration into intact oocytes but are redundant when spermatozoa are deposited in the oocyte cytoplasm by means of ICSI. Indeed, in ICSI spermatozoa are deposited in oocytes with the acrosome still intact. The sperm acrosome contains a variety of hydrolytic enzymes whose release into the oocyte cytoplasm might cause harmful effects. The acrosome size varies considerably among mammals; humans have relatively small acrosomes, which contrasts with some rodent species such as the hamster that represent the opposite extreme of the scale.[1] The small size of the human acrosome, together with a large size of the human oocyte compared with that of the spermatozoon (Fig. 1), contributed to the ease with which human ICSI was developed with a relatively simple micromanipulation technique.[5,6] By contrast, ICSI in the mouse and some other rodents demands special techniques and instruments.[7] No special treatment of human spermatozoa is necessary to remove the sperm acrosome before ICSI,[8] whereas ICSI results are substantially improved by artificial induction of the acrosome reaction in the hamster.[9]

Electron microscopic pictures of human oocytes after ICSI demonstrate that injected spermatozoa lose their acrosomes by membrane fusion and vesiculation.[10] a process that is similar, morphologically, to the physiologic acrosome reac-

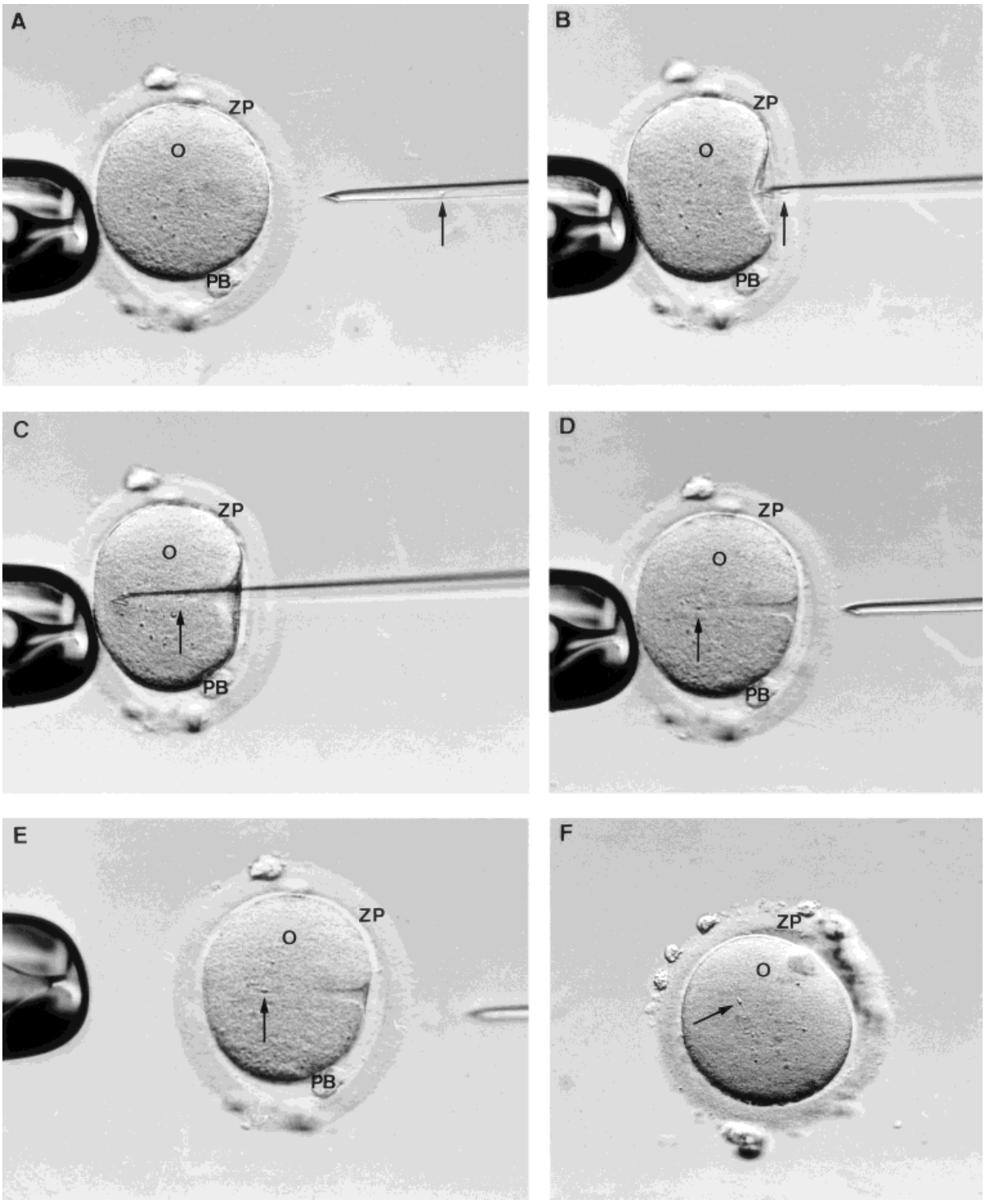

Figure 1. Sequence of micrographs (Hoffman contrast optics) showing the key-moments of human ICSI. A: The oocyte (O) is immobilized on the holding pipette (left). A spermatozoon (arrow) can be seen within the microinjection needle (right). Zona pellucida (ZP), surrounding the oocyte, and the first polar body (PB) are also visible. B: The microinjection needle is being pushed against the oocyte, and the spermatozoon (arrow) is located at the needle tip. C: The microinjection needle has been introduced deeply into the oocyte cytoplasm. D: The spermatozoon (arrow) has been deposited in the oocyte cytoplasm, and the microinjection needle has been taken out of the oocyte, leaving a clearly visible injection canal. E: The injected oocyte has been released from the holding pipette. The injection canal in the vicinity of the injected sperm head (arrow) is no longer visible. F: The same oocyte 2 minutes after ICSI. The injected spermatozoon (arrow) is clearly visible, and the injection canal has completely disappeared.

tion.[1] The physiologic acrosome reaction depends on an orderly and sequential release of individual zymogens and their conversion into active enzymes, controlled by step-wise degradation of acrosomal matrix components.[11] It is possible that a similar step-wise release of acrosomal enzymes occurs after the injection of an acrosome-intact spermatozoon into the oocyte cytoplasm. However, the activation of acrosomal enzymes from inactive zymogens requires precise conditions involving pH, removal of intra-acrosomal inhibitors, and eventual activation by specific activators. In the absence of such ideal conditions within the oocyte cytoplasm, inactive zymogens released from the acrosome may be destroyed before they can achieve enzymatic activity.

Mechanism of oocyte activation revisited

Unlike those sperm functions that serve to assist sperm penetration into intact oocytes and that are thus rendered unnecessary when ICSI is used, the ability of spermatozoa to induce oocyte activation is a crucial requirement for successful fertilization in both situations. The first activating signal is delivered to the oocyte by the fertilizing spermatozoon after binding to the oocyte plasma membrane, around the time of fusion between the plasma membranes of both gametes. This interaction induces a cascade of cell signalling events in the oocyte, driven by changes in intracellular cytosolic free calcium concentration ([Ca^{2+}]i).

Elevation of [Ca^{2+}]i is an early event in the oocyte activation cascade. In the hamster, the first [Ca^{2+}]i increase occurs as early as 10–30 seconds after attachment of the fertilizing spermatozoon to the oocyte plasma membrane.[12] It begins near the sperm attachment site, spreads throughout the oocyte, forming a Ca^{2+} wave, and ends 15–20 seconds later. The initial [Ca^{2+}]i increase in human oocytes after fusion with the fertilizing spermatozoon follows a similar pattern.[13] However, unlike most nonmammalian species, the Ca^{2+} signal that mediates the sperm-induced activation of mammalian oocytes is not limited to a single [Ca^{2+}]i transient, the first Ca^{2+} spike is followed by a series of secondary sharp [Ca^{2+}]i increases, referred to as Ca^{2+} oscillations. Sperm-induced Ca^{2+} oscillations usually last several hours after the initial [Ca^{2+}]i increase, and their frequency shows considerable interspecies variability.[14]

The first description of Ca^{2+} signals after ICSI came from studies on human oocytes.[13,15] These studies demonstrated Ca^{2+} oscillations (Fig. 2A), although their form was slightly different compared with subzonal insemination (Fig. 2B), a less invasive method of micromanipulation-assisted fertilization in which the interaction between gamete surfaces is not suppressed.[13] As natural fertilization, Ca^{2+} oscillations after ICSI also last for several hours.[16] In contrast, no Ca^{2+} oscillations were observed after sham injection of oocytes with sperm-free culture medium,[15] demonstrating the decisive role of sperm factor(s) in this process. These observations corroborated the idea that a sperm factor is responsible for oocyte activation.[17] The development of Ca^{2+} oscillations in oocytes after ICSI has now been confirmed in the mouse.[18]

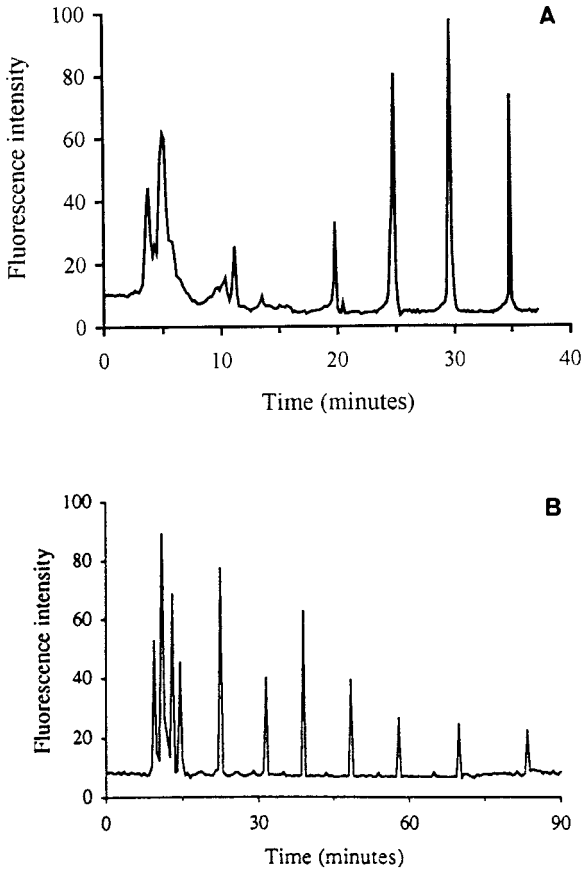

Figure 2. Ca^{2+} signals (visualized as changes in the intensity of fluorescence emitted by the Ca^{2+} indicator fluo-3) developing in human oocytes after ICSI (A) and after subzonal insemination (B). B is reproduced from Ref. 13 with permission from the American Society for Reproductive Medicine (Fertility and Sterility 1994;62:1197–1204). Oocyte loading with fluo-3 and recording of the fluorescent signals were performed as described.[13]

The molecular mechanism by which the fertilizing spermatozoon induces Ca^{2+} oscillations in the mammalian oocyte is still a matter of debate. Most investigators adhere to one of two major hypotheses on this subject and attribute the main role either to an oocyte surface receptor coupled to a G-protein-[19] or tyrosine-kinase–mediated[20] signalling pathway on the one hand, or to a direct action of a soluble sperm factor in the oocyte cytoplasm on the other.[21,22] What lessons can be drawn from the current ICSI data? The success of the human application of ICSI, together with the observation of Ca^{2+} oscillations in sperm-injected oocytes, were initially interpreted as a confirmation of the "soluble

sperm factor'' hypothesis. In fact, human oocytes can be activated by injecting intact, but not heat-treated, spermatozoa, and the removal of the injected spermatozoon 30 minutes later does not prevent oocyte activation, suggesting that a heat-sensitive factor, responsible for this process, is released to the oocyte cytoplasm by that time.[23] It has been suggested that fertilization failure after ICSI, observed in some cases of abnormal sperm development such as globozoospermia, is due to the lack of this sperm activity.[24] However, a more detailed analysis of the Ca^{2+} signals developing after ICSI in human oocytes and their comparison with those observed after conventional IVF[25,26] have questioned such a straightforward conclusion, at least in humans. The main experimental evidence against the role of a soluble sperm factor as the only stimulus responsible for oocyte activation comes from quantitative analysis of the speed of Ca^{2+} wave propagation during sequential $[Ca^{2+}]i$ increases in human oocytes.[25] These data have shown that, after conventional IVF, the first sperm-induced Ca^{2+} wave spreads from its initiation at approximately the same speed (~11µm/sec) in all directions. In contrast, subsequent Ca^{2+} waves propagate much faster around the oocyte periphery (~55µm/sec), whereas the wave speed in the centripetal direction remains unchanged. However, when oocytes are previously injected with a subthreshold dose of soluble sperm factor and subsequently incubated with spermatozoa, the selective acceleration of Ca^{2+} wave propagation around the oocyte periphery is already apparent during the first sperm-induced $[Ca^{2+}]i$ increase.[25] Thus, the action of soluble sperm factor(s) during conventional IVF is not fully developed until the second $[Ca^{2+}]i$ increase in fertilized human oocytes and is unlikely to trigger the initial sperm-induced $[Ca^{2+}]i$ increase.

These observations argue in favour of a hypothesis in which the fertilizing spermatozoon activates the oocyte by exerting distinct trigger and oscillator functions.[26] Of these two functions, only the latter, consisting of a complex of actions at intracellular Ca^{2+} stores to create an intracellular environment favourable to sustained Ca^{2+} oscillations, is exerted by a soluble sperm factor, whereas the former suggests an action of the spermatozoon at the oocyte plasma membrane just before sperm-oocyte fusion (Fig. 3). A similar mechanism has been suggested previously for mouse oocyte activation.[27] The main advantage of the trigger/oscillator hypothesis is that it explains most of the current findings concerning oocyte activation after ICSI and its abnormalities. In addition, it reconciles the soluble sperm factor hypothesis with the ''oocyte surface receptor'' hypothesis. In particular, the trigger function accommodates previous experimental findings that suggested a role for oocyte surface receptor-coupled signalling pathways in mammalian oocyte activation.[26]

Because the first sperm-induced $[Ca^{2+}]i$ increase in oocytes is mainly due to Ca^{2+} release from intracellular Ca^{2+} stores, and inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores prevail in the cortical region of mature mammalian oocytes,[28] where sperm-induced Ca^{2+} release is initiated (Fig. 3), the signal generated by the fertilizing spermatozoon at the tyrosine kinase– or G-protein–coupled receptors is likely to be transduced through activation of phosphoinositide-specific phospholipase C. Phospholipase C–catalyzed cleavage of plasma membrane phosphoinositides results in a local rise in the concentration of IP_3 and diacylglycerol (DAG), the former releases Ca^{2+} from the IP_3-sensitive stores and the latter activates protein kinase C (PKC), which is thought to cooperate with the sperm oscillator to determine the frequency and amplitude of subsequent Ca^{2+} oscillations.[26]

A recently cloned mammalian protein, termed oscillin,[17] represents a possible candidate to mediate the oscillator function. The purified sperm fraction from which this protein was cloned induced Ca^{2+} oscillations in oocytes of several mammalian species.[17] A later study,[29] however, failed to detect any Ca^{2+}-mobilizing activity in a recombinant protein prepared by using primers based on the published oscillin sequence.[17] At the same time, several other candidates for the sperm oscillator, such as a truncated c-kit receptor, which is present in mouse spermatozoa and whose microinjection into mouse oocytes induces Ca^{2+}-dependent activation-associated events[30] or proteins associated with sperm perinuclear material,[31] have emerged. The complex problem of the current search for sperm components that promote Ca^{2+} oscillations in oocytes is beyond the scope of this paper, and the reader is referred to a recent focused review on this subject for details.[32] Anyway, such components might be looked for among molecules capable of modifying the biological characteristics of two major receptors that serve as second messenger-gated Ca^{2+} channels controlling Ca^{2+} release from intracellular stores, the IP_3 receptor and the ryanodine receptor. Both IP_3-sensitive and ryanodine-sensitive Ca^{2+} channels can be opened, in addition to their specific agonist, by an increase in $[Ca^{2+}]i$ in their immediate vicinity (Ca^{2+}-induced Ca^{2+} release; CICR).[33] Hence, the ability of oocytes to sustain Ca^{2+} oscillations must be dependent on a fine coordination of the sensitivity of the two types of Ca^{2+} stores to CICR, on their ability to reabsorb the released Ca^{2+} by the action of Ca^{2+} pumps in the store membranes, on the Ca^{2+}-buffering capacity of intra-store Ca^{2+}-binding proteins, and on the distribution of individual types of stores in the oocyte cytoplasm.[34,35] Sperm factor(s), thus, may exert their oscillator function by modulating the sensitivity to CICR of IP_3-sensitive or ryanodine-sensitive Ca^{2+} stores, the former being a more likely target, at least in human oocytes in which microinjection of sperm extract selectively accelerates the speed of Ca^{2+} wave propagation around the oocyte periphery[18] where only ryanodine-insensitive Ca^{2+} stores have been detected.[34]

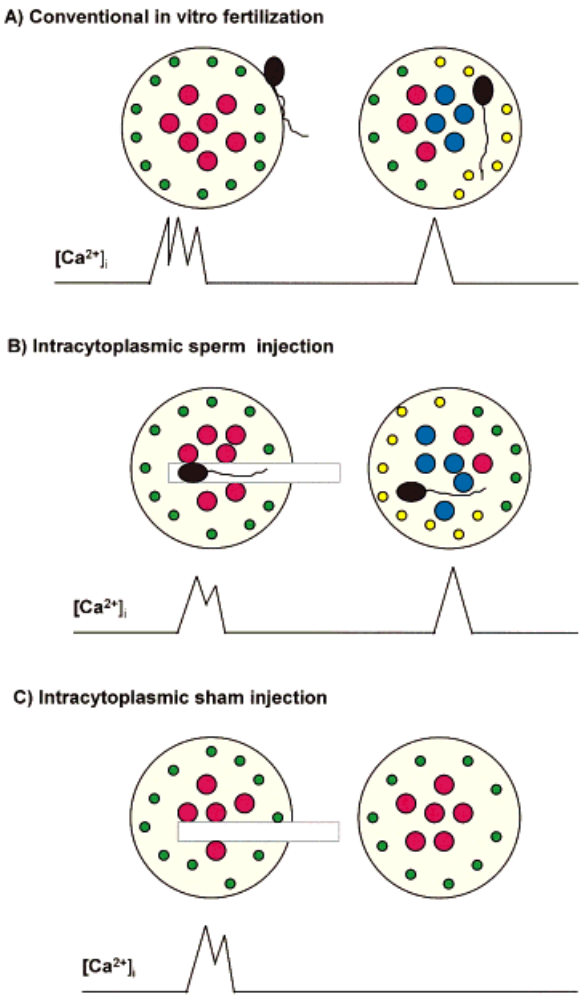

Figure 3. Schematic representation of the presumptive changes in Ca^{2+} release characteristics of the oocyte's intracellular Ca^{2+} stores resulting from different treatments and their relationship with actual changes in $[Ca^{2+}]i$. Colour changes of Ca^{2+} stores represent modifications of their sensitivity to Ca^{2+}-induced Ca^{2+} release in response to sperm factor(s), not their actual Ca^{2+} load. A: During conventional IVF, the first sperm-induced Ca^{2+} release results from the sperm trigger function, realized during sperm contact with the oocyte cell membrane, without changing functional properties of the IP3-sensitive (small circles) and ryanodine-sensitive (large circles) Ca^{2+} stores. In contrast, the second $[Ca^{2+}]i$ increase occurs spontaneously when the properties of both types of stores have been modified by sperm soluble factor(s) released to the oocyte cytoplasm. Compared with this simplified representation, a more gradual transition between these two functional states is likely to occur during this process. B: During ICSI, the sperm trigger function is by-passed, but an increase in $[Ca^{2+}]i$ is produced by the injection procedure itself (artificial pseudotrigger). The second $[Ca^{2+}]i$ increase occurs spontaneously when the properties of both types of stores have been modified by sperm-soluble factor(s) released to the oocyte cytoplasm, similarly as after conventional IVF. C: Intracytoplasmic sham injection also produces a $[Ca^{2+}]i$ increase, but the oocyte's Ca^{2+} stores do not change their properties, and no subsequent $[Ca^{2+}]i$ increase occurs.

According to the trigger/oscillator hypothesis, the development of sperm-induced Ca^{2+} oscillations requires sequential action of a trigger at the oocyte plasma membrane and of an oscillator inside the oocyte cytoplasm (Fig. 3). How is it then possible that Ca^{2+} oscillations also develop after ICSI, when all gamete surface interaction is eliminated? The answer to this question has been offered by experiments in which $[Ca^{2+}]i$ was monitored continuously in human oocytes whilst they were injected with spermatozoa.[36] In these studies, the injection procedure itself produced a significant Ca^{2+} influx into oocytes from the surrounding culture medium (along the ~10,000-fold concentration gradient between the medium and the cytosol), which led to an increase in $[Ca^{2+}]i$ similar to that resulting from sperm-oocyte fusion (Fig. 4). The lack of the sperm trigger in the instance of ICSI, thus, is overcome by an artificial pseudotrigger. As to the oscillator, the release of components exerting this function from the injected spermatozoon to oocyte cytoplasm is facilitated by previous mechanical damage to the spermatozoon. This is usually carried out by crushing the sperm flagellum with the microinjection needle against the bottom of the culture dish just before ICSI.

The current practice of human ICSI has led to the identification of abnormalities in oocyte activation that can be explained by an isolated failure of either the trigger or the oscillator functions. Selective failure of the pseudotrigger function occurs when ICSI is performed in conditions that do not allow sufficient Ca^{2+} influx from the external medium. This may occur when the surrounding medium has a reduced Ca^{2+}concentration, when ICSI is performed too rapidly or when the aspiration of the oocyte cytoplasm before injection (a usual step ensuring a correct breakage of the oocyte plasma membrane) is omitted without applying other means facilitating Ca^{2+} entry, such as repeated multidirectional movements of the microinjection needle within the oocyte.[26] In fact Ca^{2+} entry occurs spontaneously while the microinjection needle remains within the oocyte cytoplasm, but is

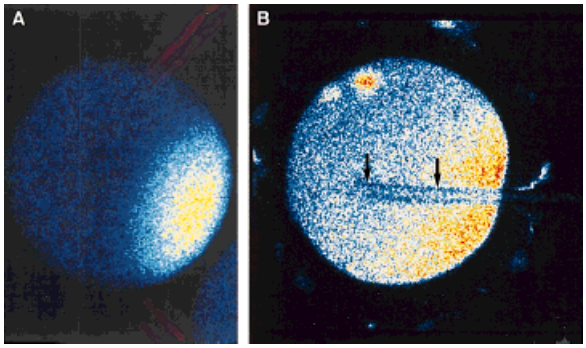

Figure 4. Confocal laser scanning microscopy images of the first $[Ca^{2+}]i$ increase occurring in human oocytes after conventional in vitro fertilization (IVF) and after intracytoplasmic sperm injection (ICSI). Oocyte loading with fluo-3 and recording of the fluorescent signals were performed as described.[42] Relative Ca^{2+} concentrations are represented in colours according to a scale in which the lowest concentrations are dark blue and the highest are red. A: After conventional IVF, the first $[Ca^{2+}]i$ increase begins at the sperm attachment site (lower right) and spreads throughout the oocyte cytoplasm as a Ca^{2+} wave. B: The first $[Ca^{2+}]i$ increase in oocytes treated by ICSI results from an influx of extracellular Ca^{2+} through the wounded cell membrane laterally from the inserted microinjection needle (arrows).

substantially enhanced by aspiration of the oocyte cytoplasm and by movement of the needle within the oocyte.[36] Trigger insufficiency does not preclude the normal function of the sperm oscillator, which can remain latent until provided with an appropriate stimulus. In this latent condition, oocytes do not show Ca^{2+} oscillations, do not complete the second meiotic division (retention of the second polar body), do not decondense the injected sperm nucleus and do not develop pronuclei. Yet, when such oocytes are subjected to treatments that increase $[Ca^{2+}]i$ sufficiently (e.g., by a short incubation with a Ca^{2+} ionophore), they can develop delayed Ca^{2+} oscillations,[37] complete meiosis, decondense sperm nuclei, and develop pronuclei.[38]

In contrast, in case of a selective failure of the oscillator function (due to inherent sperm deficiency or to delayed release of sperm factors in the ooplasm because of insufficient mechanical damage to sperm before ICSI), injected oocytes undergo a single $[Ca^{2+}]i$ rise but do not develop Ca^{2+} oscillations.[15] This condition may lead to a complete failure of oocyte activation or result in incomplete activation patterns characterized by the completion of some activation events and failure of others.

Developmental consequences of abnormal oocyte activation

Inadvertently, human ICSI has become a huge source of information about the impact of oocyte activation abnormalities on future embryonic development. This finding is attributable to several independent factors: the unusual fragility of the oocyte activation process after ICSI (substitution of the natural trigger by an artificial pseudotrigger; see above), dependence of both the trigger and the oscillator function on the quality of instruments used for ICSI, and on the micromanipulation technique (which apparently are not quite the same in different laboratories); and extensive application of ICSI in the treatment of severe male infertility, including cases with inherent abnormalities of the sperm oscillator function and, last but not least, the existence of multicentre outcome records that include the frequency of abnormal fertilization, implantation failure, spontaneous abortion, and genetic and nongenetic abnormalities of children born after ICSI.

Complete failure of oocyte activation, as evidenced by the persistence of metaphase of the second meiotic division and by the presence of condensed sperm chromatin,[39] is the most frequent cause of fertilization failure after ICSI. As mentioned above, this situation is often a sequela of insufficiency of the micromanipulation-driven compared with the natural sperm-derived oocyte activation trigger (Table 1).

The development of tripronucleate zygotes is another common fertilization anomaly after human ICSI.[40] Most of these are triploid and result from retention of the second polar body.[41] Interestingly, failure of the cell cycle progression can also be produced in the nemertean worm *Cerebratulus lacteus* by truncating the fertilization-associated Ca^{2+} oscillatory signal.[42] A comparable situation is likely to arise after human ICSI when the injected spermatozoon is deficient in cytoplasmic factor(s) that exert the oscillator function (Table 1). However, 36% of human tripronucleate zygotes that develop after ICSI have been shown to be diploid.[41] The mechanism of the formation of supernumerary pronuclei in these cases is not clear, but if the anaphase anomaly also leads to an unequal partition of homologous chromatids between the oocyte and the second polar body, abnormalities in chromosome number can arise. This mechanism has been suggested as one of the possible causes of de novo chromosomal anomalies after ICSI[43] and may be relevant to a report describing an unusually high frequency of sex-chromosome abnormalities in a small group of ICSI children.[44]

Unipronucleate zygotes are another relatively frequent fertilization anomaly after ICSI. In most such cases, mechanisms unrelated to oocyte activation, such as expulsion of the injected spermatozoon through the injection canal into the perivitelline space or sequestration of the sperm nucleus in a vacuole-like structure,[10] appear to be involved. On the other hand, failure of the male pronucleus formation after conventional IVF is likely to be caused by insufficiency of oocyte cytoplasmic factors responsible for sperm chromatin decondensation, subsequent chromatin rearrangements and nuclear envelope formation (Table 1). Experiments with human zona-free oocytes have shown that exhaustion of these factors by

TABLE 1. Principal Causes of Different Types of Developmental Failure After Conventional IVF and After ICSI*

Developmental pathology	Prevailing pathogenetic mechanism	
	Conventional IVF	ICSI
Persisting metaphase II block	Failure of sperm penetration	Failure of oocyte activation
Tripronucleate zygote	Bispermic penetration	Anaphase II failure
Unipronucleate zygote	Oocyte sperm-decondensing factor insufficiency	Sperm expulsion or sequestration in a vacuole
Embryo growth arrest	Oocyte anomaly (DNA, RNA, mitochondria)	Sperm anomaly (DNA, oscillator)
Embryo cell lineage imbalance	Oocyte anomaly (DNA, RNA, mitochondria)	Sperm anomaly (DNA, oscillator)
De novo chromosomal anomalies	Oocyte non-disjunction	Sperm anomaly (non-disjunction, oscillator)

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

increasing the number of spermatozoa competing for them in polyspermically penetrated oocytes leads to partial or complete failure of sperm nuclear decondensation in normally activated oocytes.[45] Condensed sperm nuclei cannot normally be detected in living oocytes on the day after ICSI.

In addition to fertilization anomalies, alteration of oocyte-activating signals can have developmental consequences reaching far beyond the completion of meiosis and fertilization (Table 1). In fact, these early signals can influence the speed of cell division in preimplantation mammalian embryos and cell allocation to the first embryonic cell lineages.[46,47] As in sea urchin embryos,[48] in both mouse[49] and human,[16] these events seem to be controlled by Ca^{2+} signals, which are both cell-cycle and -stage specific. Thus, it is tempting to speculate that alterations in the early fertilization-induced signal, caused by abnormalities of the trigger or oscillator function, entail subsequent alterations of signals that control early embryonic development. This relationship, however, remains to be demonstrated experimentally. It may be relevant in this context that human zygotes resulting from ICSI seem to have less resistance to environmental stress after cryopreservation. In fact, frozen/thawed ICSI zygotes cleave slower, have worse morphology, implant less easily, and have a higher risk of early abortion compared with frozen/thawed zygotes resulting from conventional human IVF.[50] It remains to be assessed whether the slight alteration of the oocyte-activating signal after ICSI[13] has anything to do with these differences.

Specific features of application in human reproductive medicine

High efficacy in most forms of male infertility

Surprisingly, the application of ICSI in human assisted reproduction has led to similar and high fertilization, implantation, and pregnancy rates in most forms of sperm abnormalities. These include extremely severe cases with only several tens of living spermatozoa in the ejaculate, for which conception with conventional IVF would be unthinkable.[51] Moreover, ICSI has been applied with great success in men suffering from azoospermia (no spermatozoa in the ejaculate) after surgical recovery of spermatozoa directly from their testis or epididymis.[52]

These successful applications gave rise to the idea that an ICSI-based technique might also be used to achieve fertilization by using haploid sperm precursor cells spermatids.[53] This idea was reinforced after the birth of mice after fertilization by electrofusion of oocytes with round spermatids[54] and by the substantial improvement in efficiency gained by replacing electrofusion with a microinjection technique.[55] The first human births after fertilization with elongated[56] and round[57] spermatids were achieved in 1995.[58] Although the success rates of assisted reproduction techniques by using round spermatids are still relatively low, probably because a high proportion of germ cells from men with severe forms of testicular failure carry apoptotic DNA damage,[59] the inclusion of in vitro culture of germ cells before their use in assisted reproduction facilitates selection of nonapoptotic cells and, thus, can overcome this problem.[60] The same in vitro culture protocol has been used with success to achieve a pregnancy and birth after transmeiotic differentiation of male germ cells that were blocked in vivo at the primary spermatocyte stage, thus extending the possibility of assisted reproduction treatment to men with premeiotic germ cell maturation arrest.[60] Together with the recent report on a term pregnancy after intracytoplasmic injection of secondary spermatocytes,[61] these data show that, despite variable success rates, ICSI-derived techniques are now available for all types of human male germ cell maturation arrest (Table 2).

Risk factors, limitations, and how they can be overcome

There is one fundamental, and often underestimated, difference between ICSI in its application in human assisted reproduction, on the one hand, and experimental ICSI (with animal gametes or with human gametes donated for research by consenting healthy individuals) on the other. That difference is the existence of a pathologic process underlying male infertility in the former instance, whereas normal gametes are used in the latter.

TABLE 2. Application of Intracytoplasmic Sperm Injection–Derived Techniques in the Treatment of Male Infertility Caused by Spermatogenesis Arrest

Stage of spermatogenesis arrest	Technique	First birth	Reference
Elongated spermatid	Intracytoplasmic injection	1995	56
Round spermatid	Intracytoplasmic injection	1995	57
Secondary spermatocyte	Intracytoplasmic injection with ploidy manipulation	1998	61
Primary spermatocyte	In vitro spermatogenesis followed by intracytoplasmic injection	1998	60

It has been well documented that, in comparison with general population, infertile men, and particularly those with the most severe forms of infertility, have a higher prevalence of abnormal karyotypes (reviewed in Ref. 62). Consequently, embryos created by means of ICSI by using spermatozoa from such men will, in all probability, also carry a chromosomal abnormality. Most chromosomal abnormalities are incompatible with complete embryonic development and lead to a spontaneous pregnancy loss, which explains the relatively low incidence of chromosomal abnormalities in children born after ICSI compared with the high frequencies of abnormal karyotypes in men enrolled in ICSI programmes[62,63] and in preimplantation embryos that develop after ICSI.[64] In fact, the only significantly elevated risk associated with ICSI concerns de novo sex-chromosome aberrations, which were detected in 9 of 1,082 (0.83%) prenatal tests in the world's largest published series,[65] compared with general neonatal population rate of (~0.21%).[66] The de novo incidence of autosomal structural aberrations (0.37%) is also slightly elevated after ICSI.[65] The mechanism by which these chromosome aberrations develop after ICSI is not known and may involve epigenetic factors, such as the deficiency of sperm factors responsible for oocyte activation or abnormalities of the sperm centrosome.[43]

As to postnatal development of ICSI children, no significant difference from the general population has been detected for major congenital malformations or major health problems at the ages of 1 year[67] and 2 years.[68] However, some controversy has emerged as to the risk of mild delays in development, for which a significant increase (both against conventional IVF and natural conception) was found by one group,[67] whereas no difference was reported by another.[68] This point awaits further clarification. In any instance, until the pathogenetic mechanism underlying de novo sex-chromosomal aberrations and the presumptive mild mental retardation occurring in some children conceived by ICSI is elucidated, all doctors can do at the moment is inform candidate patients about the existence of these risks, without any real means of prevention.

The increasing use of immature spermatozoa and sperm precursor cells in assisted reproduction also raises questions about the possible negative impact of incomplete or defective genomic imprinting, as it is not known at which stage of spermatogenesis this process occurs.[69] This risk, however, is merely hypothetical, and there is no empirical evidence for a real danger linked to abnormal genomic imprinting in human assisted reproduction.

Conclusion

In the context of current laboratory techniques applied in clinical medicine, ICSI is exceptional because of an unusually rapid application without extensive preliminary testing in animal models. This finding is partly related to the relative difficulty of ICSI with gametes of commonly used laboratory animals compared with humans. Unlike species with greater sperm-to-oocyte volume ratios or with larger sperm acrosomes, the risk of oocyte damage by the ICSI procedure is low in humans, and the manipulation is easy. In this situation, the main causes of fertilization and developmental failure after human ICSI are related to abnormalities of oocyte activation.

Studies of oocyte activation after ICSI suggest a two-step mechanism. The first step is triggered by a massive influx of Ca^{2+} into the oocyte provoked by piercing the oocyte plasma membrane by the microinjection needle. This artificial pseudotrigger can substitute for the natural oocyte-activation trigger, supposed to result from receptor-mediated Ca^{2+} release from the oocyte's internal stores after sperm action at the oocyte plasma membrane. The second step is characterized by the development of Ca^{2+} oscillations, resulting from the release of one or several sperm factors or oscillators into the oocyte cytoplasm.

Insufficiency of the artificial pseudotrigger or sperm oscillator leads not only to a complete or incomplete oocyte activation failure, but it can also cause diverse abnormalities of fertilization and early embryonic development, including aneuploidy, embryo growth arrest, and cell lineage imbalance. Transmission of genetic defects and problems of genomic imprinting are also important issues to be addressed in relation with human ICSI, especially when immature forms of human spermatozoa or sperm precursor cells are used.

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