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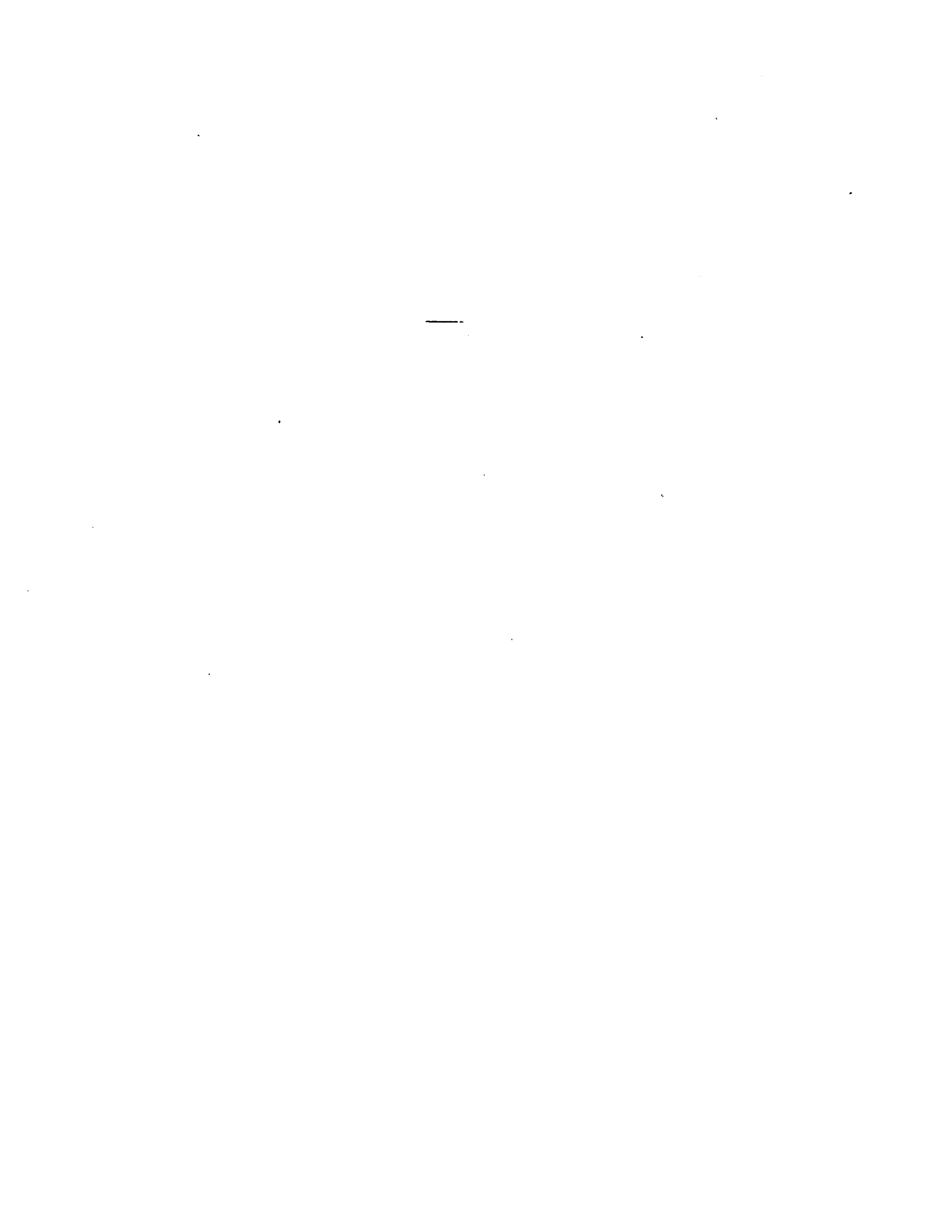

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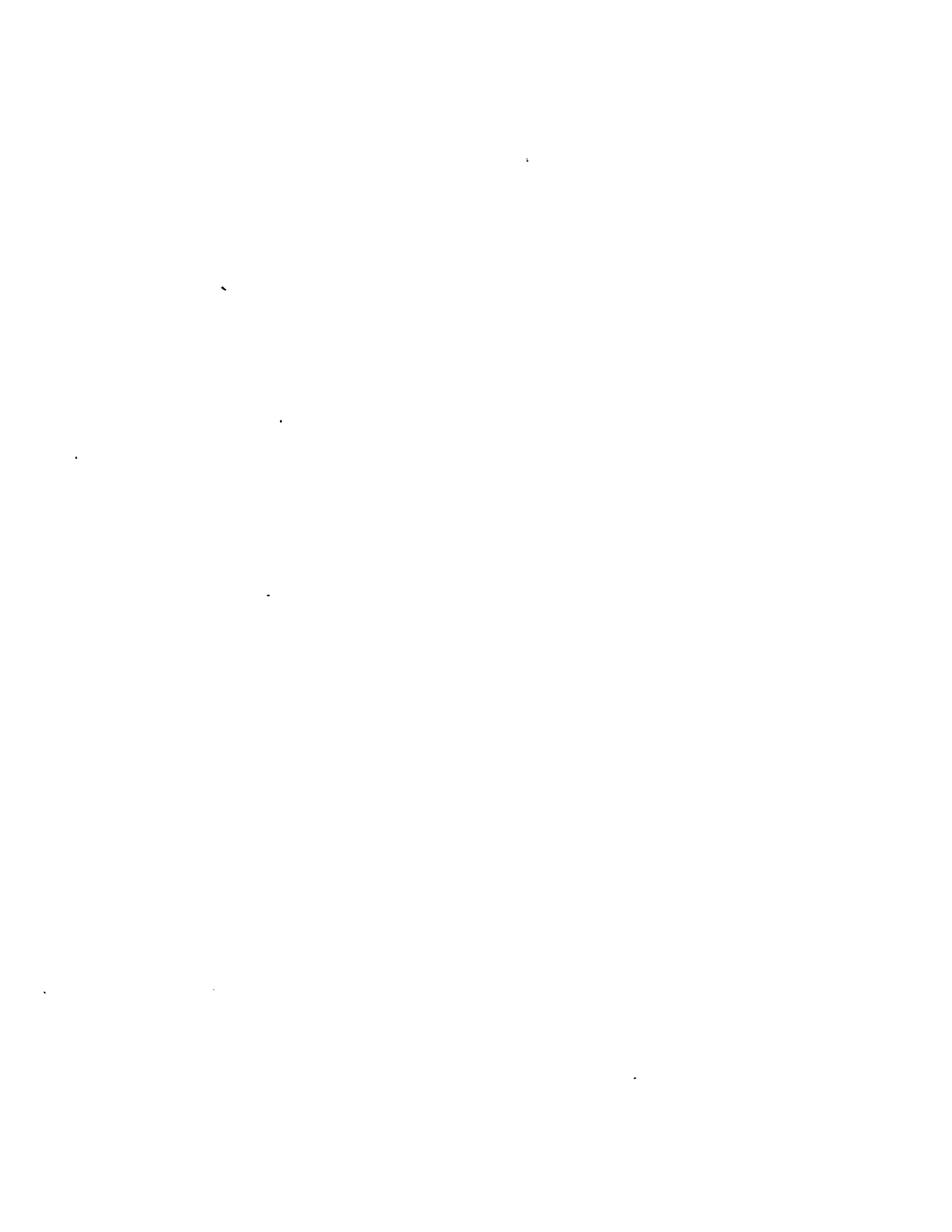

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USE OF MICROMANIPULATION AND IN VITRO FERTILIZATION TO INVESTIGATE GAMETE INTERACTIONS IN THE MOUSE

by

BETH E. TALANSKY

A dissertation submitted to the Graduate Faculty
in Biomedical Sciences in partial fulfillment of the
requirements for the degree of Doctor of Philosophy,
The City University of New York.

1988

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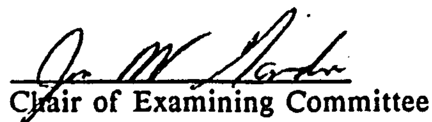

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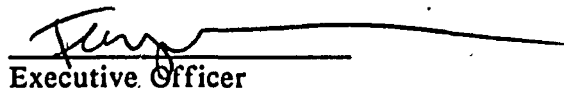

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ABSTRACT

USE OF MICROMANIPULATION AND IN VITRO FERTILIZATION TO INVESTIGATE GAMETE INTERACTIONS IN THE MOUSE

by

Beth E. Talansky

Advisor: Jon W. Gordon

In vitro fertilization and gamete micromanipulation can bypass one or more steps in mammalian fertilization and thereby allow for detailed study of the events involved in gamete fusion. In this thesis, these techniques were applied to a mouse model. Acrosome-reacted spermatozoa were inserted under the zona pellucida to determine if normal processes of sperm penetration are required for fertilization. Fusion did not occur after sperm were inserted in the perivitelline space, and the findings further suggested that mechanical manipulation of the flagellum resulted in membrane changes which rendered the sperm incapable of fertilization. To determine if these changes were based on alterations of membrane potential, sperm were exposed to Na^{+}-K^{+} ATPase inhibitors which, at the concentrations used, would not interfere with motility, but would alter membrane potential. ATPase inhibitors blocked fertilization, results which indicate that a membrane potential is required for gamete fusion in the mouse.

Because ATPase inhibitors are known to block the acrosome reaction, we induced the acrosome reaction prior to exposure. These experiments led to the novel observation that mouse sperm which have completed the acrosome reaction prior to zona binding cannot fertilize the egg.

Difficulties in fertilizing oocytes by sperm microinjection led us to devise a new approach for improving fertilization in vitro. A microneedle was used to dissolve a small hole in the zona. This "zona drilling" led to increased fertilization rates, even at reduced sperm counts. Polyspermy was rare, and transfer of embryos fertilized after drilling resulted in birth of normal live young. In a related study, oocytes were fertilized after drilling, cultured in vitro to blastocysts, and compared to controls with respect to sperm binding, time of fertilization, and patterns of cleavage development. Significant differences characteristic of the drilled embryos were observed at each stage.

In summary, the data in this dissertation identify difficulties with fertilization by sperm microinjection, elucidate the sequence of events required for sperm penetration in the mouse, indicate that the sperm membrane is important to gamete fusion, establish a new method for improving in vitro fertilization, and characterize fertilization and cleavage of eggs subjected to zona pellucida drilling.

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DEDICATION

This thesis is dedicated to my parents, Irene and Kalman Talansky whose love, emotional and academic support have enriched my life. You are my pride and joy.

TABLE OF CONTENTS

Subject	Page
Title page	i.
Copyright page	.ii.
Approval page	iii.
Abstract	.iv.
Acknowledgements	.vi.
Dedication	viii.
Table of Contents	ix.
List of Figures	xi.
List of Tables	.xii.
Introduction	1
Chapter I Capacitated, acrosome-reacted but immotile sperm, when microinjected under the mouse zona pellucida, will not fertilize the oocyte	
Abstract	19
Materials and methods	22
Results	28
Discussion	31
References cited	36
Chapter II Ion Pump ATPase inhibitors block the fertilization of zona-free mouse oocytes by acrosome-reacted spermatozoa	
Abstract	50
Introduction	51
Materials and methods	52
Results	57
Discussion	60
References cited	66
Chapter III Assisted fertilization by zona drilling: A mouse model for correction of oligospermia	
Abstract	74
Materials and methods	77
Results	79
Discussion	85
References cited	89

Subject *Page*

Chapter IV
Cleavage characteristics of mouse embryos inseminated and cultured after zona pellucida drilling

Abstract 98
Introduction 99
Materials and methods 101
Results 104
Discussion 107
References cited 112

Discussion 132

LIST OF FIGURES

Figure	Subject	Page
Chapter I		
1	Subzonal microinjection of sperm	38-39
2	Male pronucleus formation after direct microinjection of sperm	40-41
3	Electron micrographs of sperm incubated in medium, and exposed to Ca^{++} ionophore	42-43
Chapter II		
1	Electron micrograph of dbcGMP-treated spermatozoon	69-70
2	Zona-free oocytes inseminated in absence and presence of ouabain	71-72
Chapter III		
1	The zona drilling procedure	92-93
Chapter IV		
1	Zona-drilled mouse oocyte 35 min post insemination	114-115
2a-c	Zona-drilled embryos: 4-cell stage	116-119
3	Zona-drilled embryo: early compaction	120-121
4	Zona-drilled embryo: morula	122-123
5	Zona-drilled embryo: blastocyst	124-125

LIST OF TABLES

Tables	Subject	Page
Chapter I		
1	Eggs implanted after microinjection . . .	44-45
2	Eggs microinjected and examined for pronucleus formation: early experiments	46-47
	Eggs microinjected: later experiments . .	48-49
Chapter II		
1	Mouse oocytes inseminated after capacitation of sperm for 2 h or 2 h 30 min	.73
Chapter III		
1	In vitro fertilization of zona-drilled oocytes	94-95
2	Development of zona-drilled oocytes . . .	96-97
Chapter IV		
1	Fertilization and development of zona-drilled oocytes	126-127
2	Cleavage abnormalities of zona-drilled oocytes	128-129
3	Attrition rates of zona-drilled embryos during cleavage	130-131

INTRODUCTION

Mammalian reproduction involves a complex interaction between male and female germ cells at the time of fertilization. Fertilization depends upon an intricate series of maturational events of both male and female gametes, the normal physiologic function of germ cells after their release from the gonad, and appropriate timing of gamete interaction in the oviduct. For these reasons, an understanding of fertilization is important not only from a scientific point of view, but from the practical perspective that control of the process in both animals and humans is highly desirable.

The development of in vitro fertilization has allowed the study of sperm and oocyte interactions in a controlled environment. The ability to fertilize eggs in vitro also provides the opportunity to manipulate the germ cells in order to control fertility. Through such manipulations one can also gain a greater understanding of sperm and egg physiology. In this dissertation, the in vitro fertilization system is employed to perform a variety of studies of gamete function, some of them directed toward enhancing fertility. These experiments were carried out with the laboratory mouse.

The role of motility in gamete fusion

Because fertilization involves a dynamic interaction between the male and female gametes, it is not surprising that the sperm is highly specialized in its motility. However, while flagellar undulation is typically seen, sperm motility does not appear to play a major role until the sperm is in close proximity to the egg. Sperm do not actually "swim" to the site of fertilization (Bishop, 1961; Restall, 1967). Instead, propulsive oviductal fluid movements are responsible for sperm migration (Blandau, 1978; Battalia and Yanagimachi, 1979). Even the ampulla, the site of the oocyte, displays vigorous peristaltic activity which favors sperm-egg contact. These observations indicate that sperm motility becomes important only after the egg is reached, and serves to aid in egg penetration and perhaps fertilization itself.

Sperm flagellar movement becomes more pronounced after the onset of capacitation. This enhanced motility, termed hyperactivation (Yanagimachi, 1981; Olds-Clarke, 1984) is characterized by a decreased frequency in flagellar beat and a concurrent increase in flagellar lateral amplitude. The motility change may play a role in dispersing the outer cumulus complex. This is especially true in the case of young oocytes whose cumulus matrices are viscous and compact (Austin, 1961). Although not definitively shown, it is likely that highly motile sperm achieve penetration of the outer cumulus complex with greater ease.

It has been suggested that the whiplike movement typical of hyperactivation may also be important for zona penetration (Katz and Yanagimachi, 1981). Although the direction from which the sperm

approaches the zona varies among different mammalian species (Austin, 1961; Yanagimachi, 1966; Sato and Blandau, 1979; Yanagimachi, 1981) it is always necessary for the sperm head to make adequate physical contact with the egg investments in order for penetration to occur. Thus, it is likely that the active thrusting movements which propel the sperm up against the zona are those which help achieve this contact.

Until the point of sperm-egg contact, the role of motility is relatively understood. However, after the sperm reaches the egg plasma membrane, the importance of motility is not clear. It has been reported (Aitken et al., 1983) that immotile human sperm from individuals with Kartagener's Syndrome, a genetic disease affecting the flagellar ATPase, are capable of penetrating zona-free hamster oocytes. This suggests that immotile sperm possess the ability to fertilize zona-free oocytes. However, such cross-species fertilization may simply represent an absorption process, rather than a fusion between male and female gametes representative of true fertilization. In _in vitro_ studies using immobilized sperm, zona-free mouse oocytes were not readily fertilized (Wolf and Armstrong, 1978). This could be due to inadequate contact between sperm and egg which normally is achieved through sperm movement. In order to evaluate the fertilizing capacity of immotile sperm, it would be helpful to utilize techniques to bring the two membrane surfaces into direct contact.

As part of this thesis, we tested the importance of sperm motility to fertilization. We used micromanipulation to bring immobilized sperm into immediate contact with the vitellus of zona-intact oocytes. In these experiments, individual immotile sperm were inserted under the zona and the eggs were then observed for signs of fertilization.

Because mammalian sperm first must become capacitated (Austin, 1951; Chang, 1951) and must undergo the acrosome reaction (Austin and Bishop, 1958) before they can fertilize oocytes, we had to be sure that prior to micromanipulation, sperm were adequately prepared for fertilization. Thus, sperm were capacitated by incubation and then induced to undergo the acrosome reaction (AR) prior to subzonal insertion. Sperm were induced to undergo the AR by a variety of artificial means. That the AR was successfully achieved was verified by electron microscopy (EM), and the ability of treated sperm to fertilize eggs prior to immobilization was confirmed by fertilization of zona-free oocytes. Therefore, aside from their being immotile, these sperm should have retained their fertilizability. Immobilization was accomplished either by incubation in high concentrations of Ca^{++} ionophore, exposure to EDTA, cold treatment or mechanical manipulation. Then, individual sperm were picked up with a microneedle and placed into the subzonal space in such a way as to create tight contact with the egg surface. As an additional control, sperm were injected directly into the oocyte. This was done in order to assure that the immobilization had not rendered the sperm incapable of forming pronuclei once entering the cell. This experimental approach therefore obviated the need for sperm motility. By assessing the ability of the immobilized sperm to fertilize oocytes, we attempted to define the role of sperm motility in the fusion process. These experiments are reported in Chapter II.

The notion that motility might be an integral part of sperm-egg fusion suggests that fusion could be an ATP-dependent process. The maintenance of energy and ion balance in the flagellum as well as in

the sperm plasma membrane appears to involve neurotransmitter-mediated transduction and ion-flux change (Bavister et al., 1976; Nelson et al., 1980; Nelson et al., 1982). While it has been shown that such mechanisms exist, it would be of interest to study the functional relationship between energy dependent ion pumps and the sperms' ability to fertilize.

The immobilization experiments in this thesis research could prove quite useful in examining this issue. First, if immobilization of sperm resulted in a failure to observe fertilization, it would suggest that motility is closely related to sperm-egg fusion. Also, if manipulations rendering the sperm immotile also affect membrane ion pumps we might have evidence of the need for such pumps in order for a sperm to be functional.

In another series of experiments, these ideas were tested. Acrosome-reacted sperm which retained their motility and fertilizability were exposed to ion pump inhibitors such as ouabain, at concentrations that would not interfere with motility. By exposing these sperm to zona-free eggs, we were able to determine if functional Na^+-K^+ ATPases were important to the ability of acrosome-reacted sperm to fertilize eggs. Since ouabain has been shown to prevent the AR in the hamster (Mrsny and Meizel, 1981), sperm were again induced to undergo the AR by artificial methods. Results from this study are reported in Chapter II.

The acrosome reaction and the sequence of events leading to fertilization

The temporal relationship of the acrosome reaction, binding of

sperm to the zona pellucida and zona penetration, all leading to fertilization, appears to vary among different mammalian species. Rat (Kuzan et al., 1984), guinea pig (Huang et al., 1981), and hamster (Cherr et al., 1986) sperm may acrosome react before binding to the zona, while mouse sperm (Saling et al., 1979; Florman and Storey, 1982; Storey et al., 1984) appear to acrosome react after zona binding. Studies of the mouse system which suggest that binding precedes the AR fall into three categories. First, investigators have studied the state of the acrosome during sperm interaction with the zona (Saling et al., 1979). Results suggest that zona binding occurs prior to the acrosome reaction. Second, 3-quinuclidinyl benzilate (QNB) a muscarinic antagonist, has been shown to inhibit the AR which occurs after zona binding (Florman and Storey, 1982). Although the reasons are unknown, sperm treated with QNB fail to fertilize zona-intact oocytes. A third type of study using calcium ionophore A23187 to induce the AR in mouse sperm indicate that if prematurely acrosome-reacted, sperm will not even bind to the zona pellucida (Saling and Storey, 1979).

While these studies all suggest that sperm must be acrosome-intact when they first bind to the zona, none definitively show that sperm cannot penetrate the zona after undergoing a premature AR. The time course observational studies did not test for fertilization. In experiments which investigated the use of QNB and Ca^{++} ionophore to study sperm-egg interaction, it was not possible to rule out that these compounds exerted non-specific effects. For example, these treatments might have inhibited fertilization by in some way altering the sperm or egg membranes. Or, QNB and Ca^{++} ionophore might have affected the

structure or function of the zona pellucida so as to prevent sperm penetration. Since these possibilities were not fully investigated by demonstrating that zona-free eggs could be fertilized in the presence of these substances, the results are inconclusive.

Accordingly, as discussed in Chapter II, experiments were conducted in which the sequence of events from the acrosome reaction to fertilization was studied in physiological terms. We acrosome-reacted mouse sperm and then tested their ability to penetrate the zona only after assuring that these cells still maintained full fertilizing ability.

Ca^{++} ionophore has been widely used for inducing the AR (Green, 1978). Its mechanism of action is to increase calcium influx, the main requirement for the acrosome reaction (Yanagimachi and Usui, 1974). However, as has been recognized previously (Talbot et al., 1976; Green, 1978), the ionophore, at high concentrations, causes an overflow of calcium into cell which must be removed by ATP. The cell thus becomes depleted of a crucial factor needed for flagellar motility. While the sperm become acrosome-reacted, they do so at the expense of their ability to move. This may be undesirable if one is interested in maintaining a highly competent population of sperm.

While motility changes at low concentrations of Ca^{++} ionophore are not always observed, it is not possible to rule out that subtle changes in membrane characteristics might be triggered by the sudden influx of calcium. Therefore, it is important to test the ability of such treated sperm on zona-free eggs, where there exists no significant barrier to sperm penetration and thus where normal sperm should be able to fertilize. Then, the use of substances such as the ionophore on

zona-intact oocytes would be more meaningful.

In order to avoid the abovementioned hazards potentially associated with the use of Ca^{++} ionophore, another protocol was used to uniformily acrosome-react a population of sperm, while still allowing them to maintain full fertilizability. Since medium supplemented with 12 mM dibutryal cyclic GMP (dB cGMP) and 10 mM imidazole was previously shown to induce the AR in guinea pig sperm (Santos-Sacchi and Gordon, 1980) we applied this treatment on mouse sperm. The sperm were first capacitated in the appropriate culture medium and then exposed to GMP-supplemented medium for an additional incubation period. Electron microscopy was used to assess that this treatment resulted in a high percentage of acrosome-reacted sperm. That the sperm thus treated maintained full fertilizability was evaluated by the insemination of zona-free oocytes. In vitro fertilization with zona-intact eggs was then conducted in order to see if sperm induced to undergo premature acrosome reactions could still penetrate the zona pellucida. To assure that the conditions necessary for in vitro fertilization were adequate, untreated control sperm were used to inseminate zona-intact eggs. This protocol was then used to define the sequence of events from the AR to subsequent fertilization of the mouse oocyte.

Increasing the efficiency of in vitro fertilization by manipulation of the zona pellucida

Millions of sperm are deposited in the female reproductive tract at the time of fertilization, although only one ultimately fuses with the oocyte. It is necessary to limit the fusion to a single sperm per egg in order to maintain a normal genetic complement for the zygote. When

polyspermy occurs, the concepti are genetically abberant and die as early embryos (Wilson, 1928).

The different mechanisms for preventing polyspermy vary among organisms (Gould-Somero and Jaffe, 1984; Jaffe and Gould, 1985). First, after successful penetration by one sperm, others might be blocked at the level of the extracellular coat, the zona pellucida. Here, egg activation causes cortical granules in the ooplasm to fuse with the plasma membrane, thus releasing protease-like substances which cause the zona to become resistant to sperm penetration (Gwatkin et al., 1973). Second, while more than one sperm may be permitted to penetrate the zona layer, the plasma membrane allows only one to gain access to the egg cytoplasm. Finally, there is physiological polyspermy, in which several sperm can enter the oocyte, but once inside, only one can actually fuse with the egg nucleus and contribute genetically to the zygote (Piko, 1961; Yu and Wolf, 1981).

In mammals, various combinations of blocks at the zona or plasma membrane have been observed. When ova were recovered from hamster, dog and sheep after in vivo matings, the perivitelline space contained no sperm (Braden et al., 1954; Austin, 1955; Austin and Braden, 1956). Thus, in these species, the zona apparently blocks polyspermy. In vitro fertilization of zona-free hamster eggs yielded polyspermic fertilization. This observation indicates that in the hamster, the zona blocks polyspermy, but the plasma membrane offers no significant barrier (Barros and Yanagimachi, 1972). In the rabbit, supernumerary sperm have been seen in the perivitelline space of fertilized ova (Braden et al., 1954). Therefore, in this case, the zona is uninvolved in polyspermy prevention, and presumably, the block takes place only at

the plasma membrane.

Mouse ova have a block at both the level of the zona pellucida and the plasma membrane. The development of the zona barrier correlates with changes in receptors. The glycoproteins which compose this extracellular zona matrix are involved in this aspect of polyspermy prevention (Bleil and Wassarman, 1980). ZP-3, a zona component possessing sperm receptor activity, decreases in activity after the egg is fertilized. In its "new" form, $ZP\text{-}3_f$, it can no longer bind sperm, or trigger the acrosome reaction, another of its functions (Bleil and Wassarman, 1980). Recently it has been shown that the sugar moiety of ZP-3 serves as the sperm receptor. Fertilization triggers the loss of this sugar, N-acetyl-D-glucosamine, and thus accounts for the decrease in sperm binding (Florman and Wassarman, 1985).

The plasma membrane barrier in the mouse is not well understood. Unlike the plasma membrane block to polyspermy in organisms such as the sea urchin (Jaffe, 1976; Jaffe and Gould, 1985) there is no apparent electrical block involving changes in membrane potential (Jaffe et al., 1983). Several other mechanisms have been proposed, including the loss of receptors for sperm at the membrane and glycoprotein additions to the oocyte membrane which create a physical barrier between the gametes (Jaffe and Gould, 1985). Though these mechanisms are speculative, it is clear that changes occur upon fertilization which lead to a plasma membrane block to polyspermy.

The barrier to sperm presented by the zona can be an insurmountable obstacle in cases of oligospermia. Here, the numbers of sperm may be so low that fertilization becomes impossible. Standard in vitro

fertilization techniques, in which sperm are exposed at high concentrations to a completely intact zona might still be ineffective, because fertilization in vitro takes place under suboptimal conditions. On the other hand, complete removal of the zona would be undesirable as a remedy for oligospermia for two reasons. First, the zona helps prevent polyspermy and it would be dangerous to rely on the plasma membrane alone to limit the entrance of supernumerary sperm. Second, the zona may serve yet uncharacterized functions important for the establishment of pregnancy (Thadani, 1982).

As reported in Chapter III of this thesis, we utilized micromanipulation to create small holes in the zona pellucida of mouse oocytes in order to facilitate sperm entry. To efficiently achieve local dissolution of zona glycoproteins, acid Tyrode's solution (Nicolson et al., 1975) was loaded into a microneedle. Then, positive flow and mechanical pressure were used to dissolve small holes in the zona. These "drilled" oocytes were than inseminated in vitro and their fertilization was studied. If the eggs fertilized normally, we repeated the procedure using low numbers of sperm as a model of oligospermia. To assure that conditions for in vitro fertilization were satisfactory, all inseminations were compared with control experiments in which intact oocytes were used. In addition, we studied the rates of polyspermy and cell death resulting from the micromanipulation procedure. If pronuclei were observed after drilling, we transferred the embryos into pseudopregnant females. Thus, we were able to study the developmental potential of the oocytes inseminated after zona drilling. This method of micromanipulation was

tested for its potential to rescue individuals with severe cases of oligospermia in which sperm counts are prohibitively low for fertilization. In addition, this method was used to determine if small manipulations of the zona affect its ability to block polyspermy and/or to support normal development.

Cleavage development of oocytes fertilized after zona drilling

If zona drilling leads to increased rates of fertilization with accompanying low rates of polyspermy, and the birth of normal live young following embryo transfer, then it may have potential use as a therapy for human infertility. With this application in mind, zona-drilled mouse embryos were studied in vitro for abnormalities of preimplantation development.

The violation of the intact zona pellucida might have subtle effects on embryo cleavage which may not necessarily be reflected in a lower rate of birth of zona-drilled embryos. Transfer of manipulated embryos at the pronuclear stage, as was done in our first study, would not reveal such effects. Therefore, the animal experiments on the development of zona drilling were redesigned to more closely approximate the protocol followed in human in vitro fertilization where concepti are cultured for at least two days before transfer. Studies were thus conducted in which manipulated embryos, instead of being transferred to the oviducts of foster females, were cultured in vitro until the blastocyst stage of cleavage. The drilled oocytes were compared with controls in terms of patterns of sperm binding at the

time of insemination, time and extent of fertilization, and their cleavage patterns as they developed to blastocysts. This allowed us to detect aberrations of preimplantation development which though not associated with reduced birth rates after transfer at the pronuclear stage, might have clinical significance. For example, the potential of zona-drilled embryos to undergo twinning or aggregation to form genetic chimeras cannot be tested by immediate embryo transfer, but can be evaluated by studying cleavage in vitro. These studies are described in Chapter IV of this dissertation.

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CHAPTER I

Capacitated, acrosome-reacted but immotile sperm, when microinjected under the mouse zona pellucida, will not fertilize the oocyte*

ABSTRACT We have devised a procedure for mechanically inserting intact, acrosome-reacted spermatozoa under the mouse zona pellucida, and have examined the ability of sperm so inserted to fertilize the mouse oocyte. Sperm immobilized by a variety of different methods are unable to fertilize the egg, despite the fact that electron microscopy confirms that they are acrosome-reacted. Control experiments show that the oocytes are capable of being fertilized by motile sperm after the microinjection procedure, and that the immobilized sperm are able to form male pronuclei after injection directly into the cytoplasm. These results indicate that in addition to its importance for penetration of egg investments, sperm motility is required for fusion of the gametes. Alternatively, the findings suggest that the enzymatic machinery required for sperm motility is very similar to that utilized for gamete fusion, and that destruction of one is likely to lead to inactivation of the other.

*Barg, P.E., M.Z. Wahrman, Talansky, B.E., and J.W. Gordon (1986) Capacitated, acrosome-reacted but immotile sperm, when microinjected under the mouse zona pellucida, will not fertilize the oocyte. J. Exp. Zool., 237:365-374. Copyright © Alan R. Liss, Inc. Reprinted by permission.

The most obvious feature of the fertilizing mammalian spermatozoon is its vigorous motility. The role of sperm movement in the fertilization process is not yet entirely clear. Sperm probably do no "swim" to the site of fertilization (Bishop, '61; Restall, '67), though flagellar movement may aid in passing through the cervical mucus (Kremer, '68), maintaining spermatozoa at the site of fertilization (Braden, '53), traversing the layer of granulosa cells, or penetrating the zona pellucida (Soupart, '80). Whichever of these functions is served by sperm motility, its need would be obviated if capacitated, acrosome-reacted spermatozoa could be brought into direct contact with the egg surface. Although zona-free eggs are not readily fertilized by immotile sperm, it is not clear that adequate contact between sperm and egg is achieved simply by placing an oocyte among a group of immotile sperm.

A method for providing such contact would be of value for two major reasons. First, one could test the relationship, if any, of sperm motility to the process of gamete fusion itself. Second, animals or humans with low numbers of motile sperm could be rendered fertile. In the latter instance, retention of the zona pellucida would be most desirable, as in some organisms hatching from the zona is a prerequisite for implantation (Kane, '72; Kane and Headon, '80).

Our laboratory has devised a procedure for mechanically inserting intact mouse spermatozoa beneath the zona pellucida and establishing extensive contact between sperm and egg. The technique involved boring a hole through the zona with a microneedle, after which intact sperm could be picked up and inserted under the zona. Contact between the sperm and oolemma was obtained by inserting the sperm opposite the

first polar body, where there exists little space between the zona and egg membrane. If, despite this advantageous placement of the sperm, inadequate contact was suspected, more extensive apposition of the gametes was accomplished by pressing the sperm against the egg surface or by adding inactivated Sendai virus to the culture medium.

This technique was used to insert sperm which had been rendered immotile by four different treatments under the zona. That the sperm used were capable of fertilization prior to their immobilization was confirmed either by conducting parallel experiments wherein mouse ova were fertilized in vitro, or by examining the spermatozoa under the electron microscope and demonstrating that the preparation contained virtually 100% acrosome-reacted sperm. When the preparative procedures themselves led to immotility, the ability of the sperm to form normal male pronuclei was demonstrated by direct injection into the ooplasm.

We report here that intact but immotile sperm, when microinjected under the zona pellucida, are unable to fertilize eggs. These findings indicate a close relationship between the motility and fusion mechanisms which can be best explained by one of two hypothesis: either sperm motility is itself essential for fertilization, or it is so closely related to the fertilization process that its inactivation is likely to concommitantly destroy the ability of the sperm and egg to fuse.

MATERIALS AND METHODS

Mice used: egg and sperm recovery

CD-1 female mice were obtained from Charles River Breeding Laboratories. B6D2f1 male mice were purchased from the Jackson laboratory, Bar Harbor, Maine. Mice were maintained on a 14:10-h light-dark schedule. Six-week-old CD-1 females were induced to superovulate with 5 international units (IU) of pregnant mares' serum (Gestyl, Organon) at 4 p.m. followed 48 h later by 2.5 IU of human chorionic gonadotrophin (Pregnyl, Organon). Females were killed at 8 a.m. on the morning after mating, and the oviducts were removed into bicarbonate buffered medium (Hoppe and Pitts, '73) supplemented with bovine serum albumin and 1 mg/ml of hyaluronidase. Oviducts were opened with forceps and unfertilized eggs with follicular cells were extruded into the dish. After 1-2 min, eggs were removed and washed in 2 ml of culture medium equilibrated with 5% CO_2 in air at 37°C. The standard medium used was a modification of Hoppe and Pitts (Thadani, '82) in which sodium lactate was omitted and sodium chloride was raised to 5.97 g/l. The final measured osmolarity was 275 mOsm/kg (Thadani, '82).

Sperm were collected from the distal vasa deferentia and cauda epididymides of male mice and placed in medium containing sodium lactate (Thadani, '82) where they were incubated for 2 h. That sperm prepared in this way were capacitated and acrosome-reacted was demonstrated by placing them with oocytes whose zonae pellucidae were removed with acid Tyrode's solution. Sperm prepared in this manner rapidly fused with the oocytes, and two to six pronuclei per oocyte

were visible within 6 h.

Induction of the acrosome reaction in vitro

Although the 2-h incubation of sperm described above was shown by others (L. Fraser, personal communication) and confirmed by ourselves to induce the acrosome reaction (AR), we were concerned that this preparative procedure was not adequate for sperm microinjection. When the incubated sperm were evaluated by electron microscopy, many were acrosome-reacted, but many had not yet undergone the AR. Therefore, individual sperm chosen randomly for microinjection might still have an acrosome and consequently be unable to fuse with the oocyte. To circumvent this problem, we adopted the following strategy for inducing the AR in virtually 100% of the sperm prior to microinjection.

Calcium ionophore A23187 successfully induces the AR in vitro in guinea pig (Green, '78) and human (Russell et al., '79) sperm. We therefore adapted these methods for mouse sperm. After capacitation, the sperm were pelleted by gentle centrifugation and resuspended in 140 mM NaCl, 4 mM KCL, 4 mM HEPES, 10 mM glucose and 2 mM $CaCl_2$ pH 7.4 (Green, '78). Calcium ionophore A23187 was then added to a final concentration of 20 uM and the cells were incubated for 40-60 min at 37^oC in an atmosphere of 5% CO_2 in air.

Completion of the acrosome reaction was confirmed by four methods. First, under light microscopy, the sperm heads appeared smaller than prior to treatment. Second, the junction of the head and tail was weakened. Third, loss of the acrosome was demonstrated ultrastructurally by electron microscopy; and fourth, when microinjected directly into the ooplasm the sperm formed male

pronuclei.

Preparation of sperm for electron microscopy

After 2 h of capacitation, control and calcium ionophore treated sperm were centrifuged for 10 min and resuspended in 3 ml of Na cacodylate buffer. After centrifugation for an additional 10 min, the supernatant was removed and the pellets were suspended in 1-1.5 volumes of 2% buffered glutaraldehyde pH 7.2-7.4. After repelleting, the sperm were post-fixed in 1% OsO_4, dehydrated in ETOH and embedded in spurr's resin. Thin sections were examined on a Phillips 300 electron microscope at a voltage of 60 Kv. The percentage of sperm which were acrosome-reacted was determined by scoring at least 100 cells picked randomly at low magnification.

Immobilization of sperm for microinjection

As with guinea pig sperm, calcium ionophore treatment led to rapid loss of motility in the mouse sperm. Thus, after washing the sperm to remove this substance, no further treatment was necessary prior to microinjection. However, because simple incubation of the sperm had been previously demonstrated to induce the AR, we were interested in microinjecting sperm without calcium ionophore treatment. In these experiments, sperm were incubated for 2 h and then immobilized by incubation overnight at 4^oC, treatment with 10 mM EDTA at 37^oC for 1 h, or manipulated with the microneedle (we found that simply touching the sperm tail with the microneedle rendered the sperm immotile).

Microinjection procedure

Previous experiments wherein sperm have been microinjected into oocytes have involved insertion of sperm heads into the ooplasm (Usui and Yanagimachi, '76; Thadani, '80; Markert, '83). These protocols usually involve separation of the sperm head from the midpiece and tail by sonication (Markert, '83) and thus may be appropriate for cytoplasmic injection, we considered it inadequate for assessing the ability of sperm microinjected under the zona to spontaneously fuse with the oocyte. In particular, we were concerned that sonication would damage surface structures needed for fusion. Accordingly, we developed a method for inserting whole sperm under the zona. This technique proved more difficult than injection of heads alone because of the large size and eccentric shape of the intact spermatozoon. Nonetheless, we were able to insert large numbers of intact sperm without incurring substantial injury to the oocyte (see Results). The procedure used is described below and shown pictorially in Figure 1.

Micropipets were made by pulling glass capillary tubing (Mercer Glass Works, 90mm x 0.5 mm ID) on a vertical pipet puller (David Kopf Instruments, model 700C). The pulled pipet tip was bevelled by grinding at an angle of 21° on a Narishige rotating grinding wheel for 35-45 sec. The tip of the bevelled edge was sharpened by a second grinding stip (Thadani, '80). Holding pipets were pulled by hand from capillary tubing using a microburner and fire polished on a De Fonbrune microforge. Sperm and eggs were loaded at opposite ends of long rectangular microdrops covered with mineral oil. Individual oocytes were grasped in the holding pipet with the first polar body positioned closest to the aperture of the holding pipet. The zona was then

punctured with the microneedle. During this maneuver, continuous positive flow of medium through the microneedle tip was maintained, such that when the zona was pierced, the stream of medium pushed the oocyte away from the microneedle. This prevented accidental entry into the ooplasm (Fig. 1a). The microneedle was then advanced and withdrawn through the opening several times so as to widen the opening. When the microneedle was withdrawn, a clearly visible canal through the zona was established.

A single sperm was then drawn "tail first" into the microneedle (Fig. 1b) and introduced through the hole in the zona. Positive flow then expelled the entire spermatozoon into the perivitelline space. Because the point of sperm entry was opposite the first polar body, withdrawl of the microneedle left very little space between the zona and oolemma. As a result, the sperm was sandwiched between these structures and was pressed against the egg membrane (Fig. 1c).

When uncertainty remained that the sperm head was in full contact with the oolemma, the microneedle was adjusted slightly and pushed against the zona at a point adjacent to the canal. When this was done, the sperm was forced against the egg and held in position. The pressure of the microneedle was sufficient to create a pocket at the bottom of which resided the sperm, whose head was almost entirely surrounded by the oocyte membrane (Fig. 1d). We also performed experiments wherein 3000 units of inactivated Sendai virus was included in the microinjection medium. This greatly increased the adhesive characteristics of the sperm, and extensive contact between the gametes was invariably seen.

Assessment of oocyte survival

Death of oocytes, usually caused by accidental penetration of the ooplasm with the microneedle, was manifested by rapid and visible loss of structural integrity. To determine that oocytes which appeared to survive by gross morphologic criteria were in fact viable, we performed experiments in which all the maneuvers of microinjection were carried out but sperm were not inserted. We then removed the surviving oocytes and treated them with acid Tyrode's solution to remove the zonae. These eggs were then exposed to capacitated sperm to determine if they were still capable of being successfully fertilized. These experiments were thus used to demonstrate that the oocyte membrane was not damaged during micromanipulation in such a way as to prevent sperm-egg fusion.

Assessment of gamete fusion

Successful fertilization of oocytes by microinjected sperm was determined by two methods. First, injected oocytes were immediately implanted into the oviducts of pseudopregnant female mice. When animals were not born, the foster mothers were sacrificed and their uteri were examined for implanation sites. In other experiments, injected oocytes were incubated for several h in vitro and examined for formation of pronuclei.

RESULTS

After manipulation, both sperm and eggs have normal developmental potential

In all experiments survival of oocytes was quite high, approaching 90%. This survival rate is not reflected by the compiled data in Table 1, which includes early experiments wherein expertise with the micromanipulation equipment had not yet been achieved. Tables 2 and 3 show similar data from subsequent experiments, but break down the data so as to illustrate the high survival rate of oocytes once facility with the equipment was attained. That the surviving cells were in fact capable of fusing with normal sperm was important to determine. Had the microinjection procedure damaged the oocyte surface, failure to observe fertilization would not necessarily be attributable to the sperm. To demonstrate fertilizability of surviving oocytes, we removed the zona pellucida after microinjection and exposed the eggs to normal motile sperm. One hundred per cent of eggs so exposed (18/18) developed multiple pronuclei, results which demonstrate that failure of microinjected sperm to fertilize eggs was not due to egg damage.

We also assessed the ability of the treated sperm to form pronuclei once introduced into the oocyte. If the treatment protocols rendered the sperm incapable of producing pronuclei, failure to observe fertilization could be due not to the inability of the sperm to enter the egg, but rather, to the inability of sperm to form pronuclei after entry.

In order to make this assessment, intact spermatozoa were microinjected directly into the ooplasm. Although this procedure

killed many eggs, those which survived did show evidence of male pronucleus formation. Figure 2 demonstrates this observation, showing the entire sperm head with tail still attached within the oocyte. At the stage shown the sperm head has initiated decondensation. In subsequent stages, visibility of the tail was lost and the eggs were indistinguishable in appearance from normally fertilized oocytes.

Prior to sub-zonal insertion, the sperm were acrosome-reacted

Electron microscopic analysis confirmed previous observations that sperm incubated for 2 h under the conditions used here spontaneously undergo the AR. However, although these sperm readily fertilized eggs, we considered it possible that many, perhaps most sperm in such preparations might not have completed the AR, and that fertilization was actually accomplished by a small subpopulation of sperm. This situation would be unacceptable for our experiments, where individual sperm were picked up and inserted under the zona. If most of our sperm had not completed the AR, fertilization would be seen only rarely. We therefore studied the sperm in each experiment by examining them under the electron microscope.

Figure 3a shows that as expected, some sperm still had an intact acrosome after simple incubation, though enough were acrosome-reacted to accomplish fertilization in vitro. We estimated that 10-20% of sperm were acrosome-reacted, enough to have yielded fertilization when these sperm were chosen at random and inserted under the oocyte. However, sufficient numbers also retained an acrosome to confuse the results. We therefore treated the sperm with calcium ionophore and

evaluated them under the electron microscope. Figure 3b shows a typical sperm from such a preparation, with the AR completed. More than 95% of calcium ionophore treated sperm were acrosome-reacted.

Extensive contact between sperm and egg is established after insertion

After sub-zonal microinjection of the sperm and removal of the microneedle, the sperm were trapped between the zona and oocyte membrane. Extensive contact between the sperm and egg was readily seen, and frequently a bright halo resulting from pressure of the sperm against the oocyte was visible. When such contact was not unequivocally demonstrable, we pressed the sperm against the egg by applying pressure with the microneedle to the region of the zona overlying the sperm. This pressure was sufficiently great to create a large indentation in the oocyte (Fig. 1d). The sperm was held in this position for up to 10 min. Removal of the microneedle resulted in a gradual return of the oocyte to a spherical shape without loss of oocyte viability. In a few experiments, 3000 units of inactivated Sendai virus was added to the microdrop and accompanied the sperm into the perivitelline space during microinjection. In these experiments the sperm immediately adhered to the oocyte and could not be removed. Through these procedures we are confident that all regions of the sperm came in contact with the oocyte membrane.

Fertilization does not take place after sub-zonal insertion of sperm

To assess the ability of immotile sperm inserted under the zona to

fertilize oocytes, we first implanted microinjected eggs into pseudopregnant female mice and watched them for pregnancy. Table 1 shows that with a variety of sperm preparations, no animals became pregnant, and no implantation sites were observed when the animals were sacrificed.

For a closer evaluation of the stage at which development of microinjected eggs was interrupted, we then repeated these experiments and observed the eggs for formation of pronuclei. Tables 2 and 3 show the results, which demonstrate that regardless of the method used to immobilize sperm, no signs of fertilization were observed.

DISCUSSION

We have developed a method for inserting intact spermatozoa under the zona pellucida of the mouse oocyte, and have evaluated the ability of sperm placed under the zona to fertilize the egg. Sperm immobilized for injection by treatment with EDTA, cold, mechanical manipulation, or calcium ionophore are unable to enter the mouse egg, even when extensive measures are taken to assure sufficient contact between the gametes.

The absence of fertilization observed in these experiments could not be due to the failure of sperm to have undergone the acrosome reaction. Even in samples treated by simple incubation, a substantial number of sperm were acrosome-reacted. Random selection of sperm from such a population should have yielded many which had undergone the AR and which therefore were suitably conditioned to fertilize oocytes. In

sperm treated with calcium ionophore, virtually every cell had completed the AR, yet no fertilization was evident.

Technical problems could explain our results. We cannot rule out that manipulation of the sperm with the microneedle during insertion did not damage the sperm membrane and block gamete fusion. We consider this possibility unlikely, as minimal contact between the microneedle and the sperm takes place during microinjection. Another possibility is that inadequate contact between sperm and egg was achieved after insertion of the sperm under the zona. Several studies in a variety of animal species show that specific regions of the sperm make contact with the oocyte at the time of fertilization (Wolf and Armstrong, '78). To assure adequate contact between the gametes, we have inserted the sperm opposite the polar body, where minimal perivitelline space exists. We have also pushed the sperm against the egg surface and employed Sendai virus to facilitate contact. Our observations indicate extensive contact between sperm and egg, such that the sperm head was virtually surrounded by the egg membranes after completion of these various maneuvers. We therefore doubt that the region of the sperm specialized for fusion with the oocyte did not contact the egg surface. However, we cannot rule out this possibility entirely. It is also conceivable that the calcium ionophore used to induce the AR destroyed important surface membrane structures needed to effect gamete fusion. We are exploring this possibility by experimenting with other preparative methods which induce the AR but which do not affect sperm motility. We are currently microinjecting these sperm under the zona. to determine if they can successfully fertilize.

If technical problems do not explain the failure of immotile sperm

to fertilize eggs, then a direct role for sperm motility in the fertilization process must be considered. It is possible that in addition to aiding in penetration of the cervical barrier and egg investments, the motility mechanism somehow fosters a highly specific interaction between the sperm and egg. If this is the case, then a new and more extensive role for sperm motility in the process of conception should be entertained. It is noteworthy that the sperm remains motile until it contacts the egg surface, and rapidly losses motility only after adhering to the oocyte surface (Gaddum-Rosse et al., '84).

Another explanation for our results is that while not directly involved in gamete fusion, the sperm motility and fusion mechanisms are very similar, and inactivation of either is likely to destroy both. The sperm depends for its motility on a complex of enzymatic and cytoskeletal machinery, a central component of which is the dynein ATPase located on the flagellar microtubule. It is likely that all of the procedures used to immobilize our sperm, except for the mechanical method, destroyed activity of this ATPase. If another ATPase exists whose role is to maintain the sperm in a state of competence for fusion with the oocyte, then its inactivation would render the sperm unable to accomplish fertilization. Numerous ATPases have been located at the sperm surface (Gordon, '73, '76; Gordon and Dandekar, '77; McGrady, '79; Mrsny and Meizel, '81), though their activities in acrosome-reacted sperm have been less extensively studied. It is therefore possible that membrane ATPases are required by the acrosome-reacted sperm in order that it be capable of fertilizing the egg. We are currently exploring this possibility with histochemical studies.

Mechanical insertion of sperm under the zona or into the egg itself has the objective of greatly increasing the efficiency of fertilization. Millions of sperm are required in the human ejaculate in order for fertilization to take place in vivo. The successful introduction of a single sperm into the egg by micromanipulation would reduce the requisite number of sperm in the ejaculate from millions to one. Success of this effort would thus confer fertility upon males, both animal and human, which are infertile because of oligospermia. As alluded to previously, we have found, in agreement with other studies (Thadani, '80; Markert, '83) that injection of sperm into the ooplasm leads to formation of male pronuclei. However, in our hands, this approach is less desirable than sub-zonal insertion of spermatozoa, because egg mortality is high and activation of the oocyte does not always take place. Moreover, artificial oocyte activation leads to a variety of developmental anomalies after sperm insertion (Barg et al., manuscript in preparation). As shown by our recent experiments compiled in Table 3, sub-zonal insertion, once mastered, is quite safe and therefore applicable to valuable agricultural livestock or human beings.

The results reported here show that sub-zonal insertion of acrosome-reacted sperm does not readily lead to union of the gametes. If sperm motility is required for conception itself, then the strategy for overcoming this problem will entail insertion of motile sperm under the zona. If not, protocols for rendering the sperm immotile while not destroying their ability to fuse with the egg will be required. We are currently exploring both of these strategies.

We are aware that genetically immotile human sperm are able to

penetrate zona-free hamster oocytes (Aitken et al., '83). These findings indicate either a species difference between human and mouse spermatozoa, or that zona-free hamster penetration is not physiologically representative of true fertilization.

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LEGEND FOR FIGURE 1

Figure 1. Subzonal microinjection of sperm.

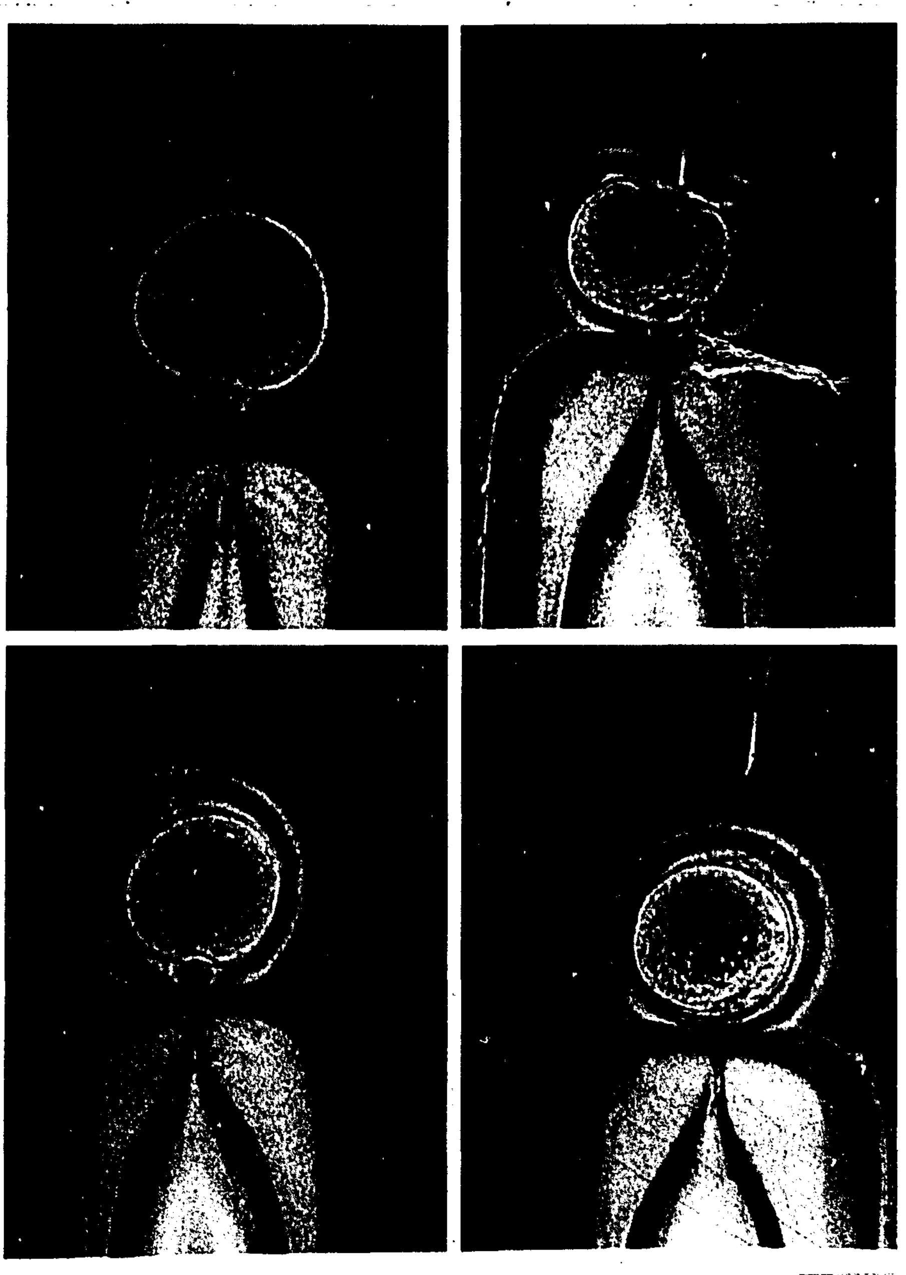
A: A hole is made in the zona pellucida while positive flow of medium through the microneedle is maintained. This flow causes the temporary increase in the perivitelline space visible here, and prevents accidental penetration of the egg with the microneedle.

B: A sperm is grasped "tail first" using suction applied to the microneedle. Here the sperm has been partially expelled to facilitate visualization. The arrow shows the sperm head.

C: The sperm is inserted under the zona. Position of the head is shown by the arrow. The perivitelline space is still slightly increased, but is contracting as excess medium is expelled.

D: The sperm is pressed against the oocyte surface by applying pressure to the overlying zona with the microneedle.

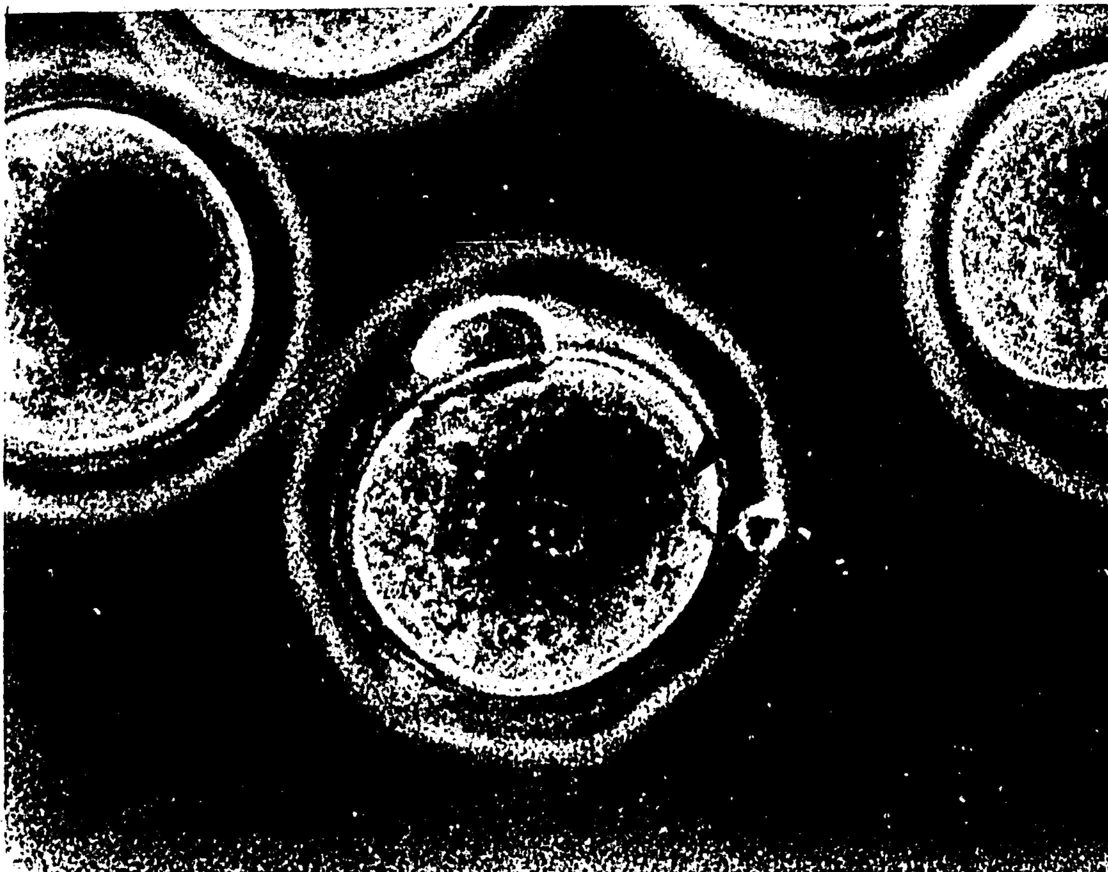
FIGURE 1



LEGEND FOR FIGURE 2

Figure 2. Male pronucleus formation after microinjection of whole sperm previously induced to undergo the AR. At the stage shown, the sperm tail is still readily visible. The arrows delimit the borders of the decondensing sperm head.

FIGURE 2



LEGEND FOR FIGURE 3.

Figure 3. Electron photomicrographs of representative samples of sperm incubated for 2 hrs. in capacitation medium (A) or exposed to calcium ionophore A23187 (B). Although many of the sperm were acrosome-reacted after simple incubation, many, exemplified by that shown in panel A, were not. Sperm exposed to calcium ionophore, were nearly 100% acrosome-reacted (panel B). Panel A: x49,000 Panel B: x60,000.

FIGURE 3

LEGEND FOR TABLE 1.

Table 1. Results of pregnancy evaluation in pseudopregnant female mice implanted with oocytes in which sperm were inserted under the zona. Survival rates are low due to pooling of data from early experiments wherein inexperience with the micromanipulation equipment led to high oocyte mortality. In later experiments survival approached 90%.

Table 1

Eggs Implanted After Microinjection

Experiment #	Treatment: Cold	Mech.	EDTA	Eggs Injected	Survived	% Survival	Females Implanted	Mice Born	Implantation Sites
4		+		204	80	39	80	0	0
12	+			857	190	22	190	0	0
4			+	367	83	23	83	0	0

LEGEND FOR TABLE 2.

Table 2. Early experiments wherein sperm were inserted under the zona and oocytes were observed for subsequent formation of male pronuclei. Survival rates were relatively low. No male pronuclei were observed.

Table 2

<u>Eggs Microinjected and Examined for Pronucleus Formation</u>
<u>Early Experiments</u>

	Treatment						
Experiment #	Cold	Mech	EDTA	Mice Injected	Mice Survived	% Survival	Male PN's
3		+		127	23	18	0
2	+			86	18	21	0
5			+	295	66	22	0

LEGEND FOR TABLE 3.

Table 3. Results of similar experiments to those tabulated in Table 2. Note the high survival rate of oocytes. Once again, no evidence of fertilization was obtained. These data include additional experiments wherein inactivated Sendai virus was employed in an attempt to facilitate sperm:egg fusion, and calcium ionophore was used to induce the AR.

Table 3
Eggs Microinjected and Examined for Pronucleus Formation

Experiment #	Treatment								
	Cold	Mech.	EDTA	Sendai	Ca Ionophore	Injected Eggs	Survival	% Survived	Male PN's
1	+					85	70	82	0
2			+			60	53	93	0
6		+				50	42	84	0
4			+	+		14	14	100	0
5					+	78	68	87	0
6			+	+	+	19	19	100	0

CHAPTER II

Ion pump ATPase inhibitors block the fertilization of zona-free mouse oocytes by acrosome-reacted spermatozoa*

SUMMARY The acrosome reaction was induced in nearly 100% of mouse spermatozoa with dibutryl cyclic guanosine monophosphate (dbcGMP) before ouabain treatment. Acrosome-reacted spermatozoa could not penetrate the zona pellucida, but readily fertilized zona-free eggs. Exposure to ouabain at concentrations as low as 10^{-7}M had a noticeable inhibitory effect upon fertilization. Similar results were obtained with a second ATPase inhibitor, digoxin. These results show that ion-pump inhibitors block the union of gametes which are otherwise fully competent to fertilize. These findings suggest that a membrane potential maintained by ion pumps is a necessary prerequisite for gamete fusion.

*Talansky, B.E., Barg, P.E., and J.W. Gordon (1987) Ion pump ATPase inhibitors block the fertilization of zona-free mouse oocytes by acrosome-reacted spermatozoa. J. Reprod. Fert. 79:447-455. Copyright © Journals of Reproduction & Fertility Ltd Reprinted with permission.

INTRODUCTION

After its release from the testis, the ejaculated mammalian spermatozoon must undergo additional changes before it is able to unite with the oocyte. Sperm must become capacitated (Austin, 1951, 1952; Chang, 1951) and complete the acrosome reaction (Austin & Bishop, 1958) before fertilizing the egg. These prerequisites have been elucidated by studies of populations of ejaculated spermatozoa. However, it is more difficult to define the precise characteristics of the single spermatozoon which reaches the egg membrane and fuses with it. This is because only one of millions of spermatozoa in the ejaculate actually fertilizes the oocyte, and because the ejaculate is a heterogenous population of cells, many of which undoubtedly never become competent to fertilize.

To assess the fertilizability of individual sperm in the mouse ejaculate we have used procedures which yield a population of uniformly acrosome-reacted spermatozoa, and have examined the ability of individual cells selected randomly from these preparations to fertilize the oocyte after mechanical insertion under the zona pellucida (Barg et al., 1986). Capacitated, acrosome-reacted spermatozoa, if rendered immotile, were unable to fertilize the egg when inserted under the zona pellucida. A striking observation in these experiments was that mechanical manipulation of the distal flagellar membrane resulted in an immediate cessation of sperm motility, suggesting that mechanical contact with the flagellum induced a change in membrane properties which then rendered the spermatozoa nonfunctional.

To test this possibility, we exposed acrosome-reacted spermatozoa

to membrane ion-pump inhibitors that would not interfere with motility but which would alter the sperm membrane potential. Previous experiments had shown that ouabain, a potent inhibitor of Na^{+}-K^{+} ATPases, interfered with sperm motility only at relatively high concentrations, and stimulated motility at low concentrations in some organisms (Nelson & McGrady, 1981). Because ouabain had also been shown to block the acrosome reaction in hamster sperm (Mrsny & Meizel, 1981) it was necessary to obtain a population of spermatozoa that was extensively acrosome-reacted before ouabain exposure. We accordingly used dibutryl cyclic guanosine monophosphate (dbcGMP) to produce uniformly acrosome-reacted, motile spermatozoa, and examined the effect of ouabain and digoxin, another Na^{+}-K^{+} ATPase inhibitor, on the ability of such sperm to fertilize the zona-free mouse oocyte.

MATERIALS AND METHODS

Mice used; egg and sperm recovery

CD-1 female mice were obtained from Charles River Breeding Laboratories. $B6D2f_1$ male mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Mice were maintained on a 14 h light: 10 h dark schedule. CD-1 females were induced to superovulate at 6 weeks of age with 5 i.u. PMSG (Gestyl: Organon, W. Orange, NJ) at 16:00 h followed 48 h later by 2.5 i.u. hCG (Pregnyl: Organon). Females were killed at 08:00 h the morning after injection and the oviducts were removed into bicarbonate-buffered medium (Hoppe & Pitts, 1973) supplemented with 4 mg bovine serum albumin/ml. Oviducts were opened with forceps and unfertilized eggs were expressed into depression

slides containing the same medium supplemented with 1 mg hyaluronidase/ml. After the granulosa cells were dispersed, the eggs were removed and washed in 2 ml of culture medium equilibratd with 5% CO_2 in air at 37^oC. The standard medium used was a modification (Thadani, 1982) of that of Hoppe & Pitts (1973) in which sodium chloride was raised to 5.97 g/l. The final measured osmolarity was 255 mosmol/kg.

After rinsing in culture medium the oocytes were collected and treated briefly with acid Tyrode's solution pH 2.3 to remove the zonae pellucidae. The eggs were then thoroughly rinsed and loaded into 20 ul microdrops under mineral oil. Medium in the microdrops was supplemented with various concentrations of ouabain; control oocytes were loaded into medium without ouabain. Spermatozoa were added to microdrops to give a final concentration of 1×10^6 motile spermatozoa/ml. Fertilization was assessed by several criteria. In most cases fertilization was obvious by the presence of at least 3 pronuclei resulting from polyspermic fertilization of the zona-free eggs. Fertilization was also manifest by the release of the second polar body and the appearance of female and male pronuclei. Spontaneous oocyte activation was distinguished from true fertilization by the observation of second polar body release, and by the fact that the male strain used in these experiments produces pronuclei with several nucleoli, while the female pronucleus has a single large nucleolus.

Preparation of spermatozoa.

Spermatozoa were expressed from each distal vas deferens and cauda

epididymis of male mice and placed in medium supplemented with 60% sodium lactate syrup (4 ul/ml). After prior incubation to allow dispersal and capacitation, spermatozoa were divided into experimental groups for further treatment as described below.

Induction of the acrosome reaction in vitro.

It has been shown by electron microscopy that incubation for 2 h is by itself sufficient for induction of the acrosome reaction in 20-30% of mouse spermatozoa (Barg et al., 1986). However, when such sperm preparations are used to inseminate eggs, the possibility cannot be excluded that other spermatozoa which undergo the acrosome reaction after insemination are those which actually fertilize the oocyte. Since ouabain, the ion-pump inhibitor used in this study, is known to block the acrosome reaction (Mrsny & Meizel, 1981; Mrsny et al., 1984) it was necessary to establish that ouabain did not prevent fertilization by blocking the acrosome reaction in spermatozoa that were not acrosome-reacted at the end of the incubation period. Accordingly, a series of experiments was performed wherein the spermatozoa were exposed to dbcGMP before ouabain treatment. The concentrations and exposure times applied to guinea pig sperm (Santos-Sacchi & Gordon, 1980) were used in the present experiments with the modification that mouse spermatozoa were preincubated to achieve optimal capacitation before induction of the acrosome reaction by dbcGMP.

After incubation for 1 h 30 min or 2 h in capacitation medium, spermatozoa were transferred to medium supplemented with 12 mM dbcGMP, and 10mM imidazole, and incubated for an additional 30 min. This

protocol was found to induce maximal acrosome reactions as demonstrated by electron microscopy.

Tests of zona penetration by dbcGMP-treated spermatozoa.

After exposure to dbcGMP, spermatozoa were tested for their ability to penetrate the zona pellucida, by performing inseminations of zona-intact oocytes retrieved after superovulation. As a test of the in vitro fertilization procedures, pairwise inseminations with untreated spermatozoa were conducted. Differences in fertilization rates after inseminations with equal numbers of treated and control spermatozoa were then compared.

Treatment with ion pump inhibitors.

After incubation in culture medium or culture medium followed by dbcGMP treatment, spermatozoa were centrifuged at 600 g for 10 min. They were then resuspended in culture medium supplemented with 10 times greater concentrations of ouabain such that the final concentration was equal to that desired for insemination. Spermatozoa were incubated in ouabain for 20 min before insemination. In a single experiment, spermatozoa were treated with 100 ug digoxin/ml for the same 20 min period and then combined with oocytes at this same concentration of digoxin.

In experiments in which spermatozoa were not exposed to dbcGMP, they were incubated for 2 h or 2 h 30 min in culture medium and 20 min in ouabain. Control groups exposed neither to dbcGMP nor ouabain were incubated for 2 h 20 min or 2 h 50 min in normal medium. Thus all sperm preparations were preincubated for the same time period before

insemination.

Assessment of sperm count and motility.

Spermatozoa were counted and their motility assessed by diluting incubated preparations and placing them on a haemocytometer. Spermatozoa were examined by visual inspection for their degree of active motility and for forward progression across the haemocytometer grid.

Preparation of spermatozoa for electron microscopy.

After capacitation, control and dbcGMP-treated spermatozoa were centrifuged for 10 min at 600 g and the pellet was resuspended in 3 ml sodium cacodylate buffer. After centrifugation for an additional 10 min, the supernatant was removed and the pellet was resuspended in a 1-1.5 ml volume of 2% glutaraldehyde pH 7.2-7.4. After further centrifugation, spermatozoa were post-fixed in 1% OsO_4, dehydrated in ethanol and embedded in Spurr's resin. Thin sections were examined in a Philips 300 electron microscope at a voltage of 60 kV. The percentage of acrosome-reacted spermatozoa was determined by direct examination and scoring of at least 100 cells under low magnification.

Tests for ouabain toxicity.

This test was conducted by first determining the minimal concentration of ouabain necessary for complete inhibition of fertilization (10^{-5} M). Spermatozoa and eggs were then preincubated with 100 times this minimal amount (10^{-3} M). The spermatozoa were washed twice by gentle centrifugation and resuspension in culture

medium. Eggs were washed by serial transfer into several dishes of normal medium. Inseminations were then carried out in normal medium as described above.

Statistical testing.

Fertilization rates were calculated as the number of oocytes fertilized divided by the number inseminated. Statistical significance of observed differences in fertilization rates was determined by square analysis using 1 degree of freedom. Differences were considered statistically significant at a P value of less than 0.05.

RESULTS

Effect of dbcGMP treatment

Exposure of mouse spermatozoa to dbcGMP effectively induced the acrosome reaction. Examination of spermatozoa by electron microscopy showed that at least 90% (95 of 100 spermatozoa examined) were acrosome-reacted. Figure 1 shows a typical spermatozoon after exposure to this compound. The acrosome reaction is completed and the normal cellular architecture is preserved. In control preparations incubated without dB cGMP, only 20-30% of the spermatozoa were acrosome-reacted.

Dibutryl cGMP did not impair either the motility of spermatozoa or their ability to fertilize zona-free oocytes. The rate of fertilization after dbcGMP treatment was not significantly different from that of control untreated spermatozoa (Table 1).

Penetration of zona pellucida by acrosome-reacted spermatozoa.

Because spermatozoa were induced to undergo the acrosome reaction before insemination, their ability to fertilize zona-intact oocytes provided a rigorous test of the importance of an intact acrosome at the time of zona contact to the fertilization process. Treatment with dbcGMP drastically reduced the fertilization rate of zona-intact mouse oocytes (4/286, 1.4%) relative to the rate obtained with untreated spermatozoa (399/1595, 25%). This result reinforces the electron microscopic finding that dbcGMP-treated spermatozoa are nearly 100% acrosome-reacted, and confirms previous suggestions (Saling et al., 1979; Florman & Storey, 1982) that otherwise normal mouse spermatozoa that have undergone the acrosome reaction before zona contact cannot fertilize oocytes.

Spermatozoa capacitated for 2 h before ouabain treatment

After 2 h of capacitation followed by 20 min of ouabain treatment, fertilization was inhibited in ouabain concentrations as low as 10^{-6}M. Of 282 oocytes 92 were fertilized (33%). This rate was significantly lower ($P \leq 0.001$) than in control inseminations without ouabain, in which the fertilization rate was 100%. Spermatozoa treated with dbcGMP proved more resistant to ouabain inhibition than spermatozoa not treated (Table 1). Thus in these experiments, dbcGMP reduced the sensitivity of spermatozoa to ouabain.

Spermatozoa capacitated for 2 h 30 min before ouabain treatment

These spermatozoa were more sensitive to ouabain than those capacitated for only 2 h. Fertilization was significantly inhibited at

10^{-6}M, with only 35% of eggs fertilized (Table 1). At 10^{-7}M the fertilization rate was 65%, still significantly lower than that of untreated spermatozoa (65% vs 96%, $P < 0.01$). Spermatozoa treated with dbcGMP were also inhibited at 10^{-7}M ouabain, with 56% of the eggs fertilized. In contrast to spermatozoa capacitated for 2 h, those capacitated for 2 h 30 min were not more resistant to ouabain than controls. At 10^{-6}M or 10^{-7}M the fertilization rate was not higher after treatment with dbcGMP than without exposure to this compound.

Ouabain blocks fertilization, but not sperm attachment.

In control inseminations spermatozoa bound rapidly to zona-free oocytes. After oocyte activation the number of bound spermatozoa was reduced. Activated oocytes could therefore be readily identified before the appearance of a second polar body or pronuclei, and a relative paucity of bound spermatozoa was evident at the time eggs were scored for fertilization (Fig. 2a). As with the controls, inseminations in the presence of ouabain were characterized by a rapid binding of numerous spermatozoa to the egg surface. However, oocyte activation never took place. Neither second polar bodies nor pronuclei appeared. At the end of the fertilization period, large numbers of spermatozoa were still seen attached to the oocyte surface (Fig. 2b).

Ouabain does not block fertilization via non-specific toxic effects upon the gametes.

As a test for ouabain toxicity, experiments were conducted wherein spermatozoa or eggs were preincubated in ouabain adjusted to

concentrations at least 100 times greater (10^{-3}M) than those needed to inhibit fertilization totally, and 10000 times greater than concentrations needed to impair fertilization noticeably. They were then washed and inseminated in normal medium. After treatment of spermatozoa in this manner, 133/137 (97%) of zona-free eggs were fertilized, while treatment of eggs followed by washing resulted in 43/43 (100%) fertilization rate. These high concentrations therefore had no inhibitory effect upon spermatozoa or eggs.

It was not possible to rule out effects of ouabain on oocytes as well as spermatozoa. That ouabain has an effect on spermatozoa was demonstrated by the observation that spermatozoa preincubated for 2 h were more resistant to 10^{-3}M ouabain than were those capacitated for 2 h 30 min. We were unable to demonstrate a direct effect on the oocyte because oocytes were fertilized normally after washing and insemination regardless of the protocol of ouabain exposure.

DISCUSSION

We have used dbcGMP to induce the acrosome reaction in nearly 100% of mouse spermatozoa and have demonstrated that such spermatozoa are normal in their ability to fertilize oocytes. This acrosome reaction induction protocol thus contrasts with calcium ionophore treatment (which renders spermatozoa immotile) and allowed separation of the inhibitory effects of ouabain on acrosome reaction induction from its potential action upon gamete fusion itself. The results presented show that, when exposed to very low concentrations of ouabain,

acrosome-reacted spermatozoa are unable to fertilize zona-free mouse oocytes. Because in other systems inhibitors such as ouabain lead to alterations in membrane potential, these findings suggest that the gamete membranes require a potential in order for fusion to take place.

It was important in these experiments to establish that the spermatozoa were acrosome-reacted before ouabain treatment. Previous work with the hamster has demonstrated that ouabain will block the acrosome reaction and thereby indirectly block fertilization (Mrsny and Meizel, 1981). It is well established that incubation of mouse spermatozoa in appropriate media for 1-2 h induces the acrosome reaction in sufficient numbers of spermatozoa for fertilization to take place (Rogers, 1978; Thadani, 1982). The present experiments agree with these data, showing that spermatozoa incubated for 2 h 20 min and then exposed to zona-free oocytes readily fertilize the egg. (Table 1). However, examination of mouse spermatozoa by electron microscopy (Barg et al., 1986) has demonstrated that, in these preparations, only 20-30% of the spermatozoa are acrosome-reacted. When such preparations are used for in vitro fertilization, it is not possible to determine whether the 20-30% of spermatozoa that are acrosome-reacted at the time of insemination are the same spermatozoa which fuse with the oocyte. Many additional spermatozoa could undergo the acrosome reaction after insemination and fertilize the ovum. This problem has been encountered previously in experiments examining the effect of trypsin inhibitors on fertilization of zona-free mouse oocytes. Although these compounds blocked fertilization, it was not possible to rule out that such blockage resulted from interference with the acrosome reaction (Wolf, 1977).

The present study has solved this ambiguity by treating spermatozoa with dbcGMP before insemination. Electron microscopic examination has shown that at least 90% of the spermatozoa are acrosome-reacted after dbcGMP treatment. Further, the acrosome reaction induced by dbcGMP is physiologically normal. Zona-free inseminations clearly demonstrate that spermatozoa treated with dbcGMP fertilize oocytes as efficiently as do spermatozoa treated by incubation alone. Because only 5-10% of spermatozoa retain their acrosomes after dbcGMP exposure, the results argue strongly that ouabain did not block fertilization by interfering with the acrosome reaction.

It was also necessary to demonstrate that ouabain did not block fertilization via non-specific toxic effects. This possibility was tested by preincubating eggs and spermatozoa in concentrations of ouabain 10000- fold greater than that needed to inhibit fertilization. Although the spermatozoa were slowed in their motility by exposure to these high concentrations, after washing they fertilized oocytes with an efficiency equal to that of control spermatozoa. Similar findings were obtained by preincubation of oocytes. Ouabain therefore does not permanently injure the gametes, even at concentrations far in excess of those needed to block fertilization.

Although the best recognized action of ouabain is inhibition of Na^+-K^+ ATPases (Garrahan & Glynn, 1967; Jorgensen, 1982), and it does inhibit these enzymes in human (Peterson & Freund, 1973) bull (McGrady, 1979), and boar (Uesugi & Yamazoe, 1966) spermatozoa, the drug has other effects. Ouabain inhibits glycolysis in human spermatozoa at concentrations as low as 2×10^{-6}M (Peterson & Freund, 1973), and lowers flagellar beat frequency and amplitude of

bull spermatozoa at concentrations of ouabain greater than 10^{-6}M (McGrady, 1979). However, 10^{-6}M ouabain also reduces the membrane potential from -8.0 to -5.6 mV after 20 min, an incubation time identical to the preincubation employed in our experiments (McGrady, 1979). Thus while motility is changed slightly in bull spermatozoa at 10^{-6}M ouabain, membrane potential is significantly altered.

That ouabain blocks fertilization by ion pump inhibition in the present system is supported by limited studies with another Na^{+}-K^{+} ATPase inhibitor, digoxin. At a concentration of 100 ug/ml this substance blocked fertilization in 18/18 zona-free oocytes exposed to capacitated, acrosome-reacted spermatozoa. Because these drug concentrations are very high, the full set of controls performed with the ouabain studies were not extended to those with digoxin. The cumulative data support the idea that ion pump inhibition with consequent alteration of membrane potential is the cause of the fertilization block.

If ouabain blocks fertilization by ATPase inhibition, it should be possible to demonstrate ATPase activity at the cell surface. Tests for ATPase activity at the surface of the unfertilized mammalian egg have not to our knowledge been conducted. Although ouabain-sensitive ATPases have previously been localized to the periacrosomal segment of the plasmalemma in cauda epididymal rabbit and guinea pig spermatozoa (Gordon, 1977), similar attempts to localize ATPases in acrosome-reacted spermatozoa have not been made. The use of dbcGMP to produce homogeneous populations of functionally normal, acrosome-reacted spermatozoa has permitted us to undertake such studies. Preliminary results of these experiments demonstrate an

ATPase at the surface of acrosome-reacted spermatozoa which is inhibited by ouabain (unpublished observations).

When inseminations were carried out after preincubation for 2 h 20 min, spermatozoa treated with dbcGMP were less sensitive to ouabain than were controls. This suggests that the spermatozoon is one site of action of ouabain, though it again does not rule out effects on the oocyte. Even after 2 h of capacitation, spermatozoa treated with dbcGMP were more resistant to ouabain than were untreated spermatozoa. The reason for this difference is not known at this time.

In the presence of ouabain, spermatozoa bind avidly to the egg membrane but do not activate development. As shown in Fig. 2, extensive sperm binding takes place, but a second polar body is not released and a female pronucleus does not form. Ouabain therefore does not prevent sperm attachment; rather, it appears to block specific interactions which lead to oocyte activation after binding has occurred.

Acrosome reaction induction as described here has a number of potential investigational and clinical applications. The ability to induce the acrosome reaction in nearly all spermatozoa of a given ejaculate should allow more detailed characterization of the acrosome-reacted spermatozoa, a cell type which is normally only a small component of a heterogenous population. In the present study, induction of the acrosome reaction was exploited to demonstrate that mouse spermatozoa must have an intact acrosome at the time of zona contact if they are to penetrate oocytes. This possibly was suggested by previous time course studies of spermatozoa bound to the zona pellucida (Saling et al., 1979; Florman & Storey, 1982; Storey et al.,

1984). The use of dbcGMP has allowed us to establish a functionally significant relationship between rentention of an intact acrosome and zona penetration.

Induction of the acrosome reaction might also confer fertility upon oligospermic males whose low sperm counts are associated with inadequate numbers of acrosome-reacted spermatozoa. Such applications would only be successful in species in which spermatozoa are able to penetrate the zona after first undergoing the acrosome reaction, or where holes placed in the zona allow direct access of spermatozoa to the oolemma (Gordon & Talansky, 1986). One advantage of dbcGMP is that the prolonged incubation required by the lysolecithin-Ficoll protocol, which also induces the acrosome reaction (Fleming & Yanagimachi, 1982), may not be necessary. We are now conducting time-course and concentration-dependency studies to characterize better the effects of dbcGMP, and determine its potential for improving the fertilizing capacity of ejaculated spermatozoa.

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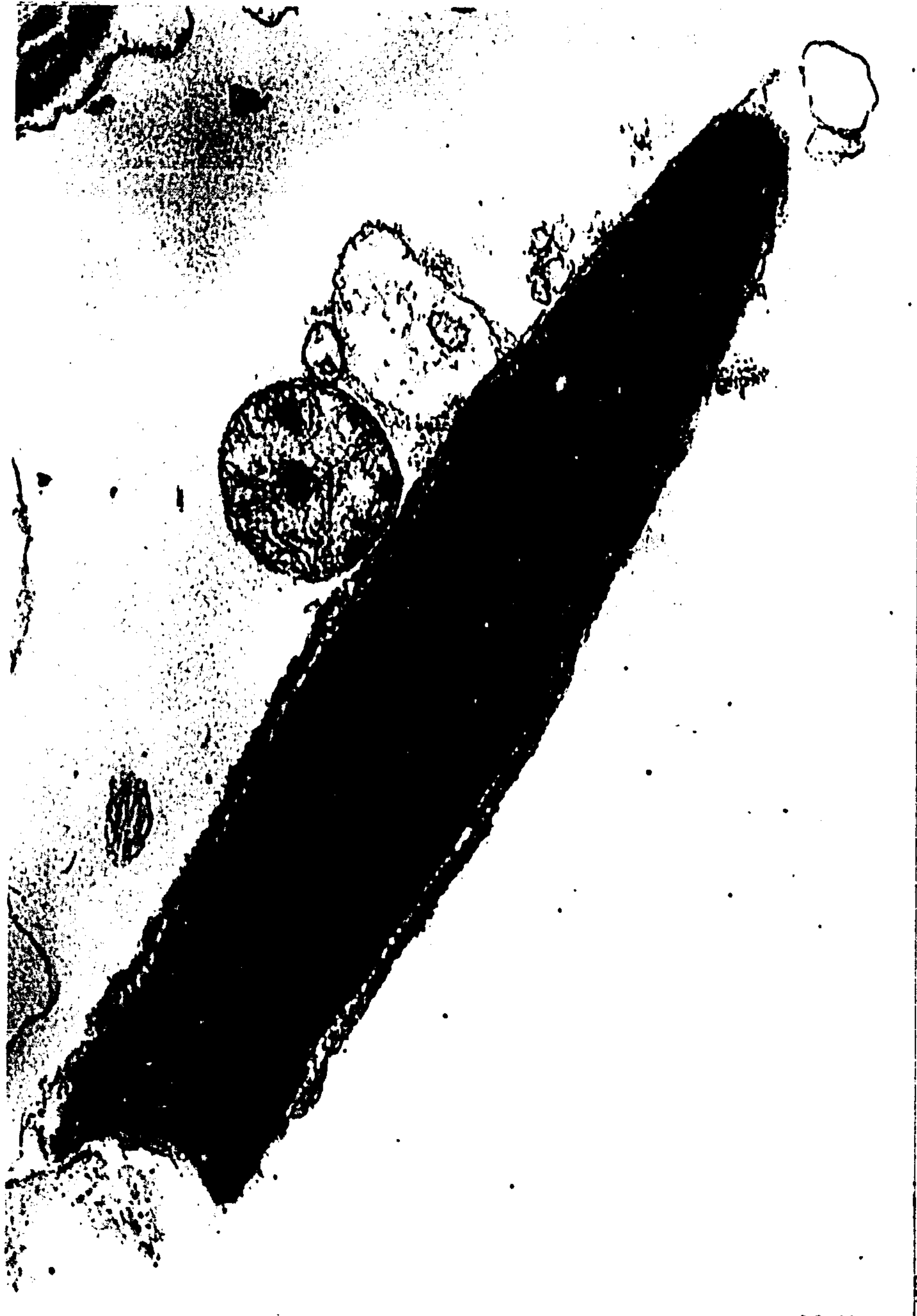
LEGEND FOF FIGURE 1

Figure 1. A typical sperm from dB cGMP treated preparations, subjected to electron microscopy. The acrosome reaction is complete. x36,000.

FIGURE 1



LEGEND FOR FIGURE 2

Figure 2. Zona-free oocytes inseminated without (panel a) or with (panel b) ouabain. At this concentration (10^{-5}) fertilization is completely blocked, but extensive binding of the spermatozoon to the vitellus still takes place (b). Signs of fertilization present in all control oocytes shown include release of the second polar body, a block to sperm binding and formation of pronuclei. Two sperm have fused with each oocyte in the control group, with resultant formation of 3 pronuclei (arrows).

FIGURE 2

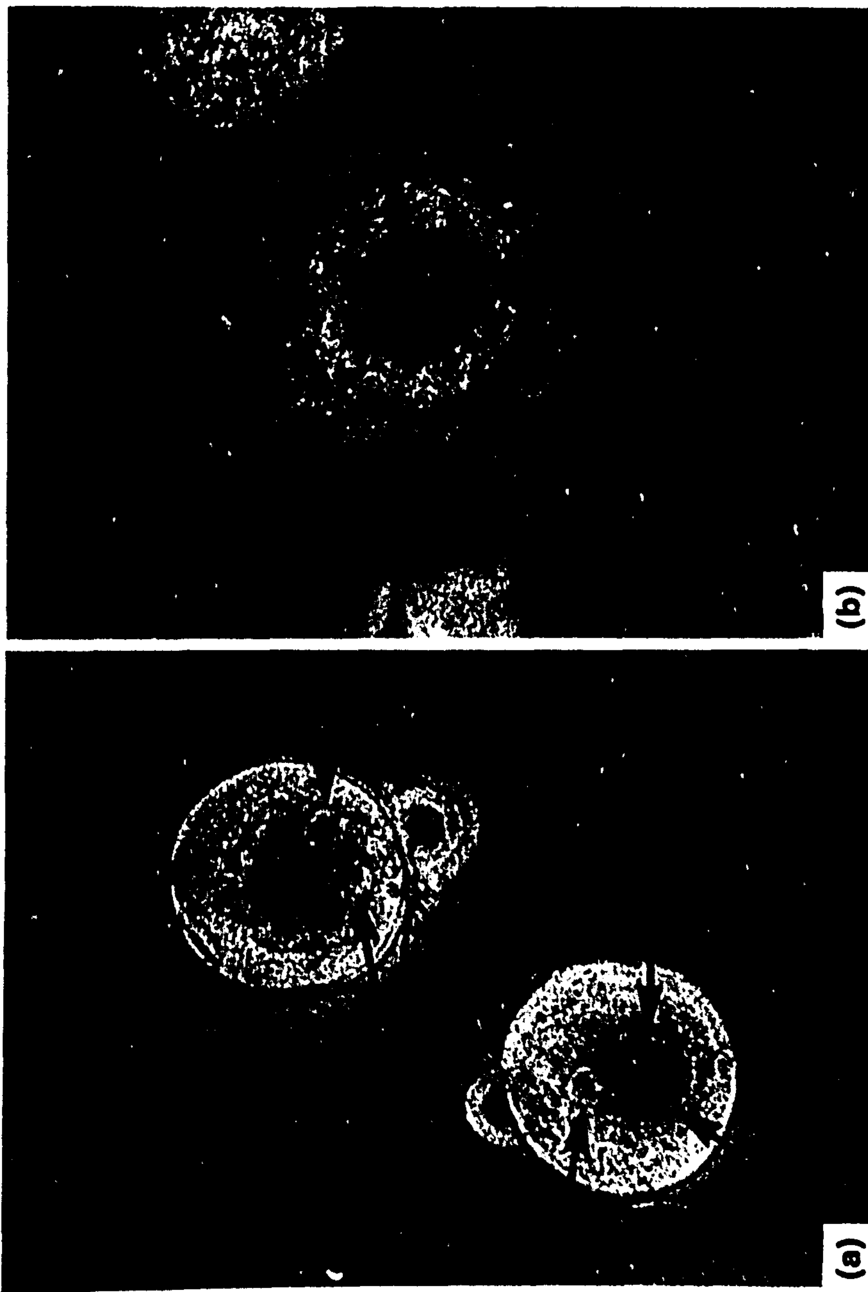


Table 1. Mouse oocyte inseminated after capacitation of spermatozoa for 2h or 2 h 30 min

Capacitation Time	Ouabain conc. (mol/l)	Ouabain	dbcGMP	No. of oocytes Fertilized/no. inseminated	(%)
2h	10^{-3}	+	−	0/8	(0)*
	10^{-4}	+	−	0/22	(0)**
	10^{-5}	+	−	1/22	(5)**
	10^{-5}	+	+	0/29	(0)**
	10^{-6}	+	−	92/282	(33)**
	10^{-6}	+	+	208/270	(77)
	0	−	−	34/34	(100)
	0	−	+	109/125	(87)
2 h 30 min	10^{-5}	+	−	0/41	(0)**
	10^{-5}	+	+	0/18	(0)**
	10^{-6}	+	−	37/105	(35)**
	10^{-6}	+	+	37/95	(39)**
	10^{-7}	+	−	46/71	(65)**
	10^{-7}	+	+	29/52	(56)**
	0	−	−	23/24	(96)
	0	−	+	19/19	(100)

*$P<0.05$; **$P<0.001$ compared with control values (x^2 test).

CHAPTER III

Assisted fertilization by zona drilling: A mouse model for correction of oligospermia*

ABSTRACT A micromanipulation apparatus was used to produce holes in the zonae pellucidae of unfertilized mouse oocytes. A microneedle loaded with acid Tyrode's solution was brought into contact with the zona surface, and positive flow was used in conjunction with mechanical pressure to cause a localized dissolution of the zona. Treated eggs were then fertilized in vitro in comparison with control cells. The zona drilling procedure decreased the sperm count required to achieve fertilization by a factor of approximately 100. The rate of polyspermy in zona-drilled oocytes was not greater than in controls, and oocytes fertilized after drilling, when implanted into pseudopregnant foster females, developed to term at the same rate as controls. The results demonstrate that zona drilling is a safe, effective method of increasing the efficiency of fertilization in vitro and may be useful both in agriculture and medicine for conferring fertility upon males with low sperm counts.

*Gordon, J.W., and B.E. Talansky (1986) Assisted fertilization by zona drilling: A mouse model for correction of oligospermia. J. Exp. Zool. 239:347-354. Copyright © Alan R. Liss, Inc. Reprinted by permission.

With respect to the male gamete, mammalian fertilization is an inefficient process. Only one sperm of millions initially deposited in the female reproductive tract actually penetrates the egg. The importance of a high sperm count to male fertility is underscored by studies of human oligospermia in which sperm counts below 2×10^7/ml of ejaculate are associated with infertility (Belsey et al., '80; Yovich and Stanger, '84). The requirement for a high sperm count is in part due to attrition of the sperm population during passage from the cervix to the site of fertilization. Once the oocytes are reached, as few as 100 sperm may remain to carry out the fertilization process (Braden and Austin, '54; Thadani, '82).

While it is not yet applicable to all mammalian species, in vitro fertilization provides one approach to correction of infertility in oligospermic males. This technique circumvents loss of sperm in the female reproductive tract and allows concentrated preparations of sperm to be placed in close proximity to the egg. In human studies, this advance has led to pregnancies in instances where sperm counts were as low as 0.5×10^6/ml of ejaculate (Jones et al., '84; Cohen et al., '85).

Although in vitro fertilization can compensate for oligospermia in some instances, it is still an imperfect technique. With rare exceptions (Bavister, '79), the ratio of sperm to eggs needed for successful fertilization in vitro is several thousand to one. In reality, fertilization in the petri dish is far less efficient than in the oviduct, where relatively few sperm are needed. That small numbers of sperm are needed at the site of fertilization in vivo suggests that it may be possible to adjust conditions for in vitro fertilization so

as to more closely approximate those found in the oviduct and thus to lower the sperm:egg ratio required for gamete fusion. This advance could confer fertility upon males with exceedingly low sperm counts. In this way, treatment of infertility could be extended to a broader population, and valuable agricultural genotypes could be more safely maintained.

Previous work with the mouse suggests that removal of the zona pellucida, a potential barrier to sperm penetration, can allow marked reduction of the sperm:egg ratio (Thadani, '82). However, removal of the zona is undesirable for two major reasons. First, this structure often contributes to the block to polyspermy (Braden et al., '54; Barros and Yanagimachi, '71), and its removal can thus lead to entry of supernumerary sperm. Second, in some species, the zona may be required for normal tubal transport or other processes necessary for establishment of pregnancy.

The experiments described in this communication were directed toward weakening the zona barrier sufficiently to lower the sperm:egg ratio without compromising the biological functions of the zona. Mouse oocytes were placed in a micromanipulation device, and acid Tyrode's solution, a potent solvent of zona glycoproteins (Nicolson et al., '75), was expelled from a microneedle, thus creating a small hole in the zona. These oocytes were then compared to zona-intact controls with respect to survival, fertilizability, susceptibility to polyspermy, and developmental potential. The findings indicate that zona dissolution may be used in both humans and animals to enhance the efficiency of in vitro fertilization.

MATERIALS AND METHODS

Mice used: egg and sperm recovery

CD-1 female mice were obtained from Charles River Breeding Laboratories; B6D2f_1 male mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Animals were maintained on 14:10 h light-dark schedule. Six-week -old females were superovulated with 5 international units (IU) of pregnant mare's serum (Gestyl, Organon, Oss, Holland) followed 48-52 h later by 2.5 I.U. of human chorionic gonadotrophin (HCG) (Pregnyl, Organon). On the morning following HCG injection, females were sacrificed and oocytes were recovered and placed in bicarbonate buffered culture medium (Hoppe and Pitts, '73), with sodium lactate omitted and sodium chloride increased (Thadani, '80; Thadani, '82). Eggs were denuded of cumulus cells by treatment with hyaluronidase, oocytes were rinsed thoroughly in culture medium and then loaded into a microdrop of HEPES-buffered M2 medium (Quinn et al., '82) for micromanipulation.

Sperm were expressed from the vasa deferentia and caudae epididymides of male mice into culture medium supplemented with 60% sodium lactate syrup (4 ul/ml). A 2 h incubation time followed to allow for sperm dispersal and capacitation.

Micromanipulation and insemination

Holding pipets for micromanipulation were fashioned by hand on a microburner and fire polished on a De Fonbrune microforge. Microneedles were pulled frdom Omega Dot tubing (Glass Co. of America, Bargaintown, NJ) using a vertical pipet puller (David Kopf Instruments,

Tujunga, CA). Manipulations were performed on a Leitz phase contrast microscope using Leitz micromanipulators.

The micromanipulation procedure is illustrated in Figure 1. Oocytes were grasped by suction with a holding pipet such that the first polar body was in the "6 o'clock" or "12 o'clock" position (Fig. 1a). The microneedle loaded with acid Tyrode's solution was then pressed against the zona in a tangential position with respect to the spherical oocyte (Fig. 1b). Acid Tyrode's solution was expelled by positive pressure in the microneedle system until a thinning, swelling, and, finally, a rupture of the zona was observed (Fig. 1c). The microneedle was then immediately withdrawn, often leaving a bleb in the oocyte surface which was exposed directly to the external environment (Fig. 1d).

After zona drilling was completed, both treated and control oocytes were inseminated in 30 ml microdrops. Motile sperm were counted using a hemocytometer and added to the microdrops to yield a final count of 10^6 or 10^4/ml in the microdrops. When 10^4 sperm/ml were used, capacitated spermatozoa were diluted with insemination medium prior to insemination. The cells were then incubated at 37°C for 6-8 h and examined for evidence of fertilization.

Assessment of fertilization

Fertilization was assessed by examining oocytes under a phase-contrast microscope. Criteria for fertilization included the presence of a second polar body and at least 2 pronuclei. In initial experiments, accurate scoring was facilitated by immobilization of eggs with 10% formalin prior to examination. When oocytes were to be

implanted, they were first evaluated for fertilization by examination under a dissecting microscope equipped with a transmitted light stage. Fertilized and unfertilized oocytes were then sorted and scored accurately in groups of ten under the phase-contrast microscope. Fertilized, diploid oocytes were segregated for implantation.

Oocytes were implanted into the oviducts of mature CD-1 female mice rendered pseudopregnant by mating with vasectomized males. Oviductal implantations were performed as previously described (Gordon et al., '80).

Statistical testing

To determine if differences between manipulated and control eggs with regard to fertilization rate, polyspermy rate, and percentage of live births were statistically significant, chi-square analyses were employed using 2 x 2 contingency tables ($\alpha = 0.05$).

RESULTS

Zona drilling is safe

Oocyte losses incurred as a result of micromanipulation fell into two categories: those killed by the procedure and those which extruded from the zona. When eggs were scored for fertilization, any deaths noted were considered to have resulted from micromanipulation. This assessment of loss tended to overestimate losses resulting directly from manipulation. Oocytes were occasionally extruded entirely from the zona encasement. Factors contributing to this "hatching" included:

increased pressure in the perivitelline space due to distortion by suction from the holding pipet, increased pressure caused by the flow of Tyrode's solution to the perivitelline space, and excessively large holes introduced in the zona by overly aggressive Tyrode's treatment. In most instances, oocytes expelled from the zona remained viable. However, because of our stated goal of retaining a biologically functional zona, these eggs were considered as lost.

Of 407 oocytes drilled, 3 were killed and 17 "hatched" from the zona. This gives a death rate of 0.7%, a hatching rate of 4%, and a total loss rate of 4.9%. The blebbing on the oocyte surface observed immediately after drilling (Fig. 1c) was not associated with oocyte mortality, and resolved spontaneously approximately 1 h after insemination. Most losses were experienced in early experiments. Later, when expertise with micromanipulation improved, we were able to substantially reduce the hatching rate. Thus, in skilled hands, zona drilling is a safe procedure.

The rate of polyspermy in zona-drilled oocytes is low

It was important to determine if violation of the zona barrier was associated with an excessive rate of polyspermy. To evaluate this issue, the rate of polyspermy in zona-drilled oocytes inseminated at 10^6 sperm/ml was compared with control eggs. No polyspermic fertilizations were observed at 10^4 sperm/ml; however, because the low sperm counts used would bias the data toward a lower rate of polyspermy, these experiments were excluded from the calculations.

All polyspermic oocytes identified were triploid. Of 289 zona-drilled oocytes inseminated at 10^6 sperm/ml, 6 were polyspermic

(2.1%; Table 1); in the control group, 4/386 (1%) were triploid. Although the incidence of polyspermy was slightly higher in zona-drilled eggs, the difference was not statistically significant by chi-square analysis ($0.25 < p < 0.5$). Thus, zona drilling does not substantially increase the frequency of polyspermic fertilization.

Zona drilling increases the efficiency of in vitro fertilization

The effect of zona drilling on the fertilization rate was evaluated from two perspectives. First, the fertilization rates in control and zona-drilled oocytes were compared using normal numbers of sperm for insemination (10^6/ml). This was done to determine if zona dissolution could improve the fertilization rate under conditions typically used in in vitro fertilization. Second, oocytes were inseminated at sperm counts normally too low for success in zona-intact eggs (10^4/ml), in order to determine if zona drilling could functionally correct infertiity due to a low sperm count. Under the conditions employed in these experiments, a sperm count of 10^4/ml is very low. Inseminations were performed in microdrops with a volume of 30 ul, and 30 eggs were loaded in each drop. Thus, the total number of sperm in each insemination drop was 300. This gives a sperm:egg ratio of 10 - far lower than is usually employed in human in vitro fertilization, in which the number of sperm used to inseminate a single oocyte may be as high as one million (Diamond et al., '85). In addition, 100-300 sperm is approximately the number of sperm which would be found at the site of fertilization in vivo (Braden and Austin, '54).

Results of these experiments are shown in Table 1. At 10^6 sperm/ml, the fertilization rate was markedly improved in the zona-drilled group as compared with the control (75% vs.22%; p<<.001). At 10^4 sperm/ml, control eggs, as expected, were not fertilized. When compared to the zero value for fertilization in the control, the 15% observed in the drilled group (Table 1) was highly significant (p <<< .001). Zona drilling therefore improves the fertilization rate at normal sperm counts and allows gamete fusion at what would otherwise be prohibitively low numbers of sperm.

The mechanism by which zona dissolution improves the efficiency of in vitro fertilization was investigated by experiments in which the sperm were induced to undergo the acrosome reaction with dibutryl (dB) Guanosine monophosphate (cGMP). Previous work had suggested that mouse sperm which undergo the acrosome reaction prior to zona contact are unable to fertiize the egg (Saling et al., '79; Florman and Storey, '82). We confirmed this finding using dB cGMP, which had been shown to induce the acrosome reaction in guinea pig sperm (Santos-Sacchi and Gordon, '80). We exposed the mouse sperm to this compound and found that nearly 100% of sperm were acrosome-reacted and that sperm thus treated, while unable to fertilize zona-free eggs, were entirely unable to penetrate eggs with intact zonae (B.E. Talansky, P.E. Barg, and J.W. Gordon, unpublished). Because of their inability to penetrate oocytes with an intact zona, these sperm could be used to probe the mechanism of enhanced fertilization in zona-drilled eggs. If sperm fertilized zona-drilled eggs by direct entry through the aperture in the zona, then dB cGMP treated sperm would fertilize zona-drilled eggs. If, in contrast, fertilization was improved by a generalized thinning or

change in structure of the zona by Tyrode's solution, dB cGMP treated sperm might not be expected to fertilize.

Accordingly, in one experiment, 19 zona-drilled oocytes were inseminated with sperm treated with dB cGMP at a count of 10^6/ml. Seventeen of these oocytes were fertilized (89%). Two controls were also performed: 40 oocytes with intact zonae were inseminated with dB cGMP-treated sperm, and none were fertilized, while 14 of 40 (35%) were fertilized after insemination with untreated sperm. In a second experiment, 112 oocytes were treated briefly with Tyrode's solution so as to expose the zona to this compound without causing complete breakdown at any point. These eggs were then inseminated with sperm treated with dB cGMP (51 oocytes) or with control sperm (61 oocytes). No fertilization was observed with sperm pretreated with dB cGMP, while 25% of eggs in the control group (15/61) were fertilized. These results show that sperm treated with dB cGMP fertilize zona-drilled eggs with high efficiency, but are unable to penetrate zona-intact oocytes, with or without prior exposure to Tyrode's solution. Thus, enhanced fertilization in the zona-drilled group is apparently not due to alteration of the zona structure by exposure to Tyrode's solution but, rather, to the complete removal of the zona barrier in the drilled area.

Zona-drilled oocytes have normal developmental potential

It was important to determine that oocytes fertilized after zona dissolution could develop normally. Two possible causes of impaired development were considered. First, exposure to Tyrode's solution could injure eggs in such a way as to block development without

noticeably affecting fertilization. Second, zona drilling could remove a biologically selective barrier which normally acts to prevent fertilization of abnormal oocytes that lack developmental potential. Either of these eventualities would be observed as a lower rate of birth, relative to control eggs fertilized in vitro, after implantation of zygotes into pseudopregnant female mice.

Accordingly, control and manipulated eggs were implanted in several experiments, and the birth rates were compared. Table 2 shows these results. Although the birth rate was slightly lower in the zona-drilled group, 36.7% vs. 44.3% in the control, the difference was not statistically significant ($0.25 < p < 0.5$). These data indicate that fertilization of a given cohort of eggs after zona drilling will lead to a higher number of live births than a corresponding group not subjected to such manipulation.

The zona pellucida retains
its biologic activity after drilling

A comparison of these data with those generated from mouse eggs that had their zonae entirely removed (Thadani, '82) demonstrates that whatever developmental role is played by the zona pellucida, that function is not significantly impaired by the drilling procedure. When the zona is removed entirely prior to fertilization, the number of implanted zygotes which develop into live young is less than 10% (Thadani, '82). In our experiments, zona-drilled eggs develop equally as well as controls. Thus, a small hole placed in zona does not impair its ability to foster normal embryo development.

DISCUSSION

Experiments reported here describe a micromanipulation technique in which a small hole placed in the mouse zona pellucida can significantly improve the efficiency of in vitro fertilization. The procedure, which entails dissolution of the zona matrix by localized application of acid Tyrode's solution, is technically simple and safe. The data indicate potential for zona dissolution for improving the reproductive potential of agricultural livestock and the clinical procedure of human in vitro fertilization.

The observation that sperm induced to undergo the acrosome reaction with dB cGMP are unable to penetrate zona-intact oocytes was exploited to study the mechanism by which zona drilling enhances fertilization. The results show that exposure of eggs to Tyrode's solution does not lead to fertilization by acrosome-reacted sperm unless that exposure is sufficient to cause a complete breakdown of the zona barrier. In experiments conducted thus far, attenuation of the zona by brief exposure to Tyrode's solution also does not increase the fertilization rate when control sperm not exposed to dB cGMP are used. Therefore, enhanced fertilization in zona-drilled eggs is probably due to sperm "swimming through the hole" made by acid Tyrode's solution. Our experiments do not rule out the possibility that exposure of the inside surface of the zona to Tyrode's solution (which occurs only during drilling) changes the zona structure, causing a generalized increase in susceptibility to sperm penetration.

Several factors must be considered when extension of this procedure to other species is contemplated. Although the mouse zona pellucida is

rapidly dissolved by acid Tyrode's solution, it is not yet clear that the zonae of other species will be similarly susceptible. We have tested the effects of Tyrode's solution on human eggs that had failed to fertilize or develop in a clinical program of human in vitro fertilization. We have found that the human zona, at least in these inviable eggs, to be more sensitive to Tyrode's solutions than that of the mouse. However, it is reasonable to anticipate that in some species the zona will be resistant to such treatment and that adjustment of the composition of the zona solvent may be necessary.

In the mouse, the rate of polyspermy was not significantly higher in treated eggs than in controls. These findings may in part be explained by the small size of the hole introduced into the zona. However, in the mouse, a rapid component of the block to polyspermy exists at the plasma membrane (Wolf, '78). In species such as the hamster, where the plasma membrane block is delayed (Barros and Yanagimachi, '72; Yanagimachi et al., '79), zona drilling could lead to a high frequency of polyspermic fertilization. One might compensate for this problem by lowering sperm counts.

Although mouse embryos conceived after zona drilling appear to develop normally, other embryos might not. While zona-free mouse embryos can develop to term (Thadani, '82), violation of the zona in other species might conceivably interfere with tubal transport, implantation, or other processes of early development. These possibilities would require consideration if zona drilling were to be extended to other animals. Finally, it must be anticipated that oligospermia will in some intances be associated with nonfunctional sperm. In these cases, zona drilling may not improve the effectiveness

of in vitro fertilization.

At a sperm count of 10^4/ml, or 10 sperm per egg, a fertilization rate of 15% was observed (Table 1). This is close to the 22% value observed for control oocytes fertilized at 10^6 sperm/ml and approximates the number of sperm found at the site of fertilization in the oviduct. It is valid to consider insemination of 30 oocytes in a 30 ul microdrop at a concentration of 10^4 sperm/ml as a sperm:egg ratio of 10, rather than as 30 eggs individually exposed to 10^4 sperm/ml. This is because the zonae of the eggs provide an extensive surface for sperm attachment, and thus act as a trap, reducing the number of freely swimming sperm available for fertilization. Moreover, because inseminations were carried out in a 30 ul microdrop, only 300 sperm were added in this group of inseminations.

The significant fertilization rate observed in experiments with 10^4 sperm/ml suggested that the sperm count might be lowered still further with a reasonable prospect of success. We have performed one experiment in which 10 oocytes were inseminated individually in microdrops with a single sperm. No fertilizations were observed, although binding of the sperm to the remaining intact portion of the zona was frequently seen. These studies suggest that in the mouse, 10 sperm per oocyte may approach a limiting dilution below which fertilization becomes highly unlikely.

One reason for this may be that many sperm, even in capacitated preparations, never undergo the acrosome reaction spontaneously (Barg et al., '86). Such sperm would of course be unable to fertilize, even if brought into direct contact with the oocyte surface. In this regard, the use of agents which induce the acrosome reaction may allow a further reduction in sperm count.

Although the utility of the zona drilling technique requires testing with other species, it appears likely that adjustment of various parameters (composition of the zona solvent, sperm numbers, and possible use of compounds that induce the acrosome reaction) could lead to significant reduction of the number of sperm required for successful fertilization in vitro. This advance would be particularly advantageous in situations where sperm must be frozen. Because freezing results in some damage to sperm, the ability to lower the count necessary for conception after sperm thawing could significantly improve the safety of freezing. Thus zona drilling, applied either by itself or in conjunction with other methodologies, could significantly improve the reproductive capacities of man and valuable agricultural animals.

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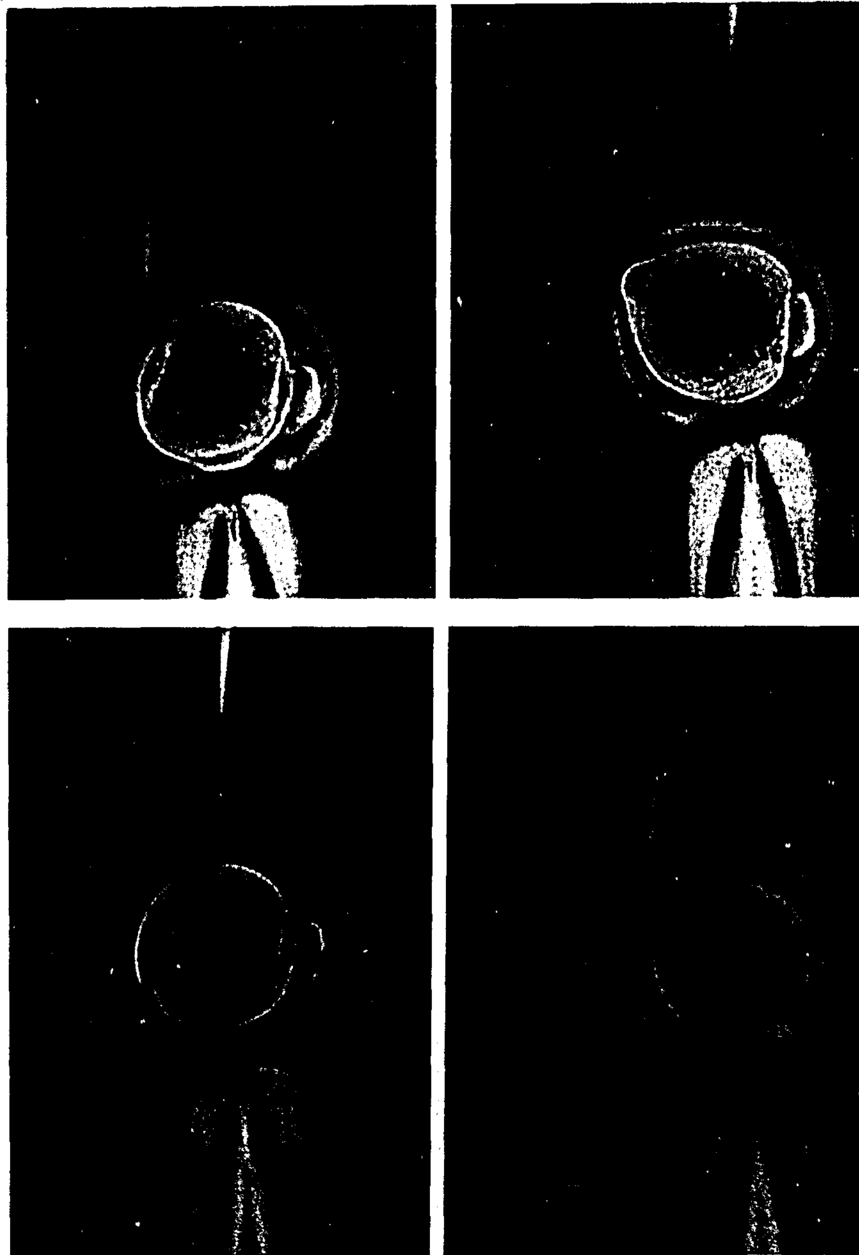
Legend for figure 1

The zona dissolution procedure. The microneedle system is prepared by loading acid Tyrode's solution into the microneedle and holding the oocyte by suction (a). The needle is placed tangentially against the zona (b), and the Tyrode's solution is expelled until the zona is locally dissolved. The opening is frequently seen when the open zona is pushed away by flow from the microneedle (c, arrow). When the procedure is completed, a small bleb appears on the egg surface and is exposed to the external environment (d).

FIGURE 1



LEGEND FOR TABLE 1.

Table 1. Efficiency of in vitro fertilization of zona-drilled oocytes compared with controls and inseminated at normal and low sperm concentrations.

TABLE 1. *In vitro* fertilization of zona-drilled oocytes

	Sperm/ml inseminated	No. oocytes inseminated	No. oocytes fertilized	Fertilized/ inseminated	No. triploid	% triploid
Drilled	10^6	289	217	75	6	2.1
Control	10^6	386	84	22	4	1.0
Drilled	10^4	118	18	15	0	0
Control	10^4	128	0	0	-	-

LEGEND FOR TABLE 2

Table 2. Rate of birth after implantation of zona-drilled and control oocytes fertilized in vitro.

TABLE 2. Development of zona-drilled oocytes

	No. embryos implanted	No. animals born	Born/ implanted (%)
Drilled	79	29	36.7
Control	61	27	44.3

CHAPTER IV

Cleavage characteristics of mouse embryos inseminated and cultured after zona pellucida drilling*

ABSTRACT The effects of zona drilling on mouse embryo development in vitro were evaluated. Following insemination, sperm were immediately concentrated at the area of drilling, and in zona-drilled eggs, pronuclei appeared 30-50 min earlier than in zona-intact controls. Zona-drilled oocytes fertilized at significantly higher rates than undrilled controls and consequently, a greater percentage of eggs inseminated after zona drilling reached the blastocyst stage. The attrition rates of zona-drilled embryos at each cleavage stage did not differ significantly from controls.

Manipulated embryos exhibited unique cleavage patterns. Some embryos lost their zonae entirely, while others became partially extruded at early cleavage stages. These anomalies led to separation of blastomeres from the zygote proper, aggregation of embryos to form giant composite morulae and blastocysts, and occasionally, to formation of miniature twin blastocysts. These characteristics of cleavage indicate that although zona drilling of a cohort of oocytes is likely to lead to an increased number of live births relative to controls, some developmental abnormalities can be encountered, and these may be associated with embryo loss, spontaneous chimerism or possibly with conception of monozygotic twins.

*Talansky, B.E., and J.W. Gordon Cleavage characteristics of mouse embryos inseminated and cultured after zona pellucida drilling. Gamete Res. In press.

INTRODUCTION

In the mouse, in vitro fertilization (IVF) is improved with zona drilling, a technique by which a microneedle loaded with acid Tyrode's solution is used to produce a small hole in the zona pellucida. Zona drilling significantly increases the efficiency of fertilization when sperm counts are reduced (Gordon and Talansky, 1986), and one-celled embryos fertilized after the procedure develop normally after oviductal transfer into pseudopregnant foster mice (Gordon and Talansky, 1986). These results indicate that fertilization assisted by zona drilling might be an effective therapy for many cases of human infertility (Gordon and Talansky, 1986), a possibility supported by results of clinical trials with infertile couples (Gordon et al., in press).

However, there exist a number of differences between zona drilling and IVF as they were performed in the mouse, and techniques employed for human IVF. In mouse experiments, fertilized ova were transferred at the pronuclear stage and were not subjected to prolonged culture as is the case for human embryos fertilized in vitro. Immediate oviductal transfer not only eliminates possible hazards of culture, it also conceals from observation any abnormalities of cleavage which might result from zona drilling. Possible abnormalities include premature hatching of the entire embryo or extrusion of portions of the embryo through the hole in the zona with consequent separation of blastomeres from the zygote. Premature hatching could create the possibility of embryo aggregation. Such aggregation, which can lead to birth of adult genetic mosaics, can be accomplished experimentally by zona removal and deliberate approximation of embryos in culture (Tarkowski, 1961; Mintz,

1962, 1965). Extrusion of parts of the embryo through the zona aperture could lead to independent development of the extruded blastomeres to yield two or more genetically identical blastocysts. When embryos are split experimentally, the likelihood that the embryo will develop into a viable conceptus may be reduced (Tarkowski and Wroblewska, 1967); alternatively, the smaller blastocysts may all develop and lead to birth of two or more genetically identical offspring (Tarkowski, 1959; Tarkowski and Wroblewska, 1967). Any of these possibilities would be of significance to patients seeking zona drilling as a remedy for infertility.

The present study was conducted to address the concern that zona drilling followed by in vitro culture might incur a risk of significant abnormalities of early cleavage development. We compared fertilization and cleavage of mouse embryos after zona drilling with the same processes in normal, zona-intact eggs. We examined the time of fertilization, the rate of development, the viability and the cleavage behavior of zona-drilled embryos in comparison to controls. Characteristics of these developmental processes unique to zona-drilled eggs are reported and described. In addition, the relevance of these abnormalities to treatment of human infertility by micromanipulation is considered.

MATERIALS AND METHODS

Media used and gamete recovery.

Sperm and oocyte recovery, and subsequent incubation of embryos, were carried out in bicarbonate buffered culture medium (Hoppe and Pitts, 1973) with sodium lactate omitted and sodium chloride increased (Thadani, 1982). All mice were of the B6D2F/1 strain obtained from the Jackson Laboratory, Bar Harbor, Me., and were maintained on a 14:10 hr. light-dark schedule. Six-week-old females were superovulated with 5 international units (IU) of pregnant mare's serum (Gestyl, Organon) followed 48-52 hr later by 2.5 IU of human chorionic gonadotrophin (hCG) (Pregnyl, Organon). Females were sacrificed by cervical dislocation 13-15 hr post hCG injection, and oocyte-cumulus complexes were recovered by puncturing excised oviducts in culture medium supplemented with hyaluronidase (1 mg/ml) in order to remove the cumulus cells. After denuding the cumulus cells, eggs which had released the first polar body were selected and loaded into microdrops of HEPES-buffered M2 medium (Quinn et al., 1982). Half of the eggs were subjected to zona drilling, while the remaining control eggs were maintained under identical conditions to the zona-drilled eggs except that they were not manipulated.

To obtain and capacitate sperm, the vasa deferentia and caudae epididymides were removed from male mice and spermatozoa were expressed into culture medium supplemented with 60% sodium lactate syrup (3ul/ml). Sperm preparations were incubated for the duration of oocyte manipulation. Therefore, the approximate period alloted for capacitation was typically 1.5-2 hr.

Micromanipulation and insemination.

The day of zona drilling and insemination was considered to be Day 1 of the experiment. The zona drilling procedure was conducted with acid Tyrode's solution loaded into a microneedle as described previously (Gordon and Talansky, 1986). Following micromanipulation, zona-drilled oocytes were thoroughly rinsed in culture medium to remove residual Tyrode's solution. Both experimental and control oocytes were then placed into 30ul microdrops under mineral oil which was saturated with insemination medium. Because of our interest in studying abnormalities of cleavage which might take place after embryo transfer (2-cell to blastocyst stage), oocytes which had completely lost their zonae as a result of drilling were considered lost and were not inseminated. Motile sperm were counted in a hemocytometer, and oocytes were inseminated at a final sperm concentration of 10^6/ml. Inseminated ova were incubated at 37°C in 5% CO_2 in air and examined periodically for pronucleus formation and second polar body extrusion, and the time elapsed between insemination and the occurrence of these events was recorded. After signs of fertilization were clearly observed in both drilled and control groups, the zygotes were placed in clean 30ul microdrops for overnight incubation.

Evaluation of fertilization and in vitro culture.

On the morning after insemination fertilization rates were assessed by counting 2-cell embryos. Embryos which were not zona-free were transferred to fresh medium (2 ml) and incubated for 5 days. Embryos which had lost their zonae at the 2-cell stage were counted but not cultured further. Each morning the development of the remaining

embryos was assessed and records were maintained of all cleavage patterns.

Statistical testing.

The differences in fertilization and rates of development between manipulated and control oocytes were determined with chi-square analysis using 2 X 2 contingency tables (α=0.05).

RESULTS

For each day of in vitro manipulation and culture, features unique to the zona-drilled eggs and embryos are described.

Day 1: Insemination and pronucleus formation.

Immediately upon insemination, sperm avidly bound to the entire periphery of the oocytes. However, among the manipulated oocytes, sperm became concentrated at the area of the hole produced by drilling. This pattern of binding was evident as early as 10 min after insemination and persisted for several hours (Fig. 1). Although the time required for pronucleus formation varied somewhat between experiments, pronuclei and second polar bodies consistently appeared 30-50 min earlier in the zona-drilled eggs. This difference was not associated with significantly more rapid cleavage or blastocyst formation.

Day 2: 2-cell stage.

Of 913 zona-drilled oocytes, 711 cleaved to 2 cells (78%). This

rate was significantly higher (p <<.001, Table 1) than in the control group (440/847, 52%). Of the 2-cell embryos in the drilled group, 34/711 were zona-free (5%) and 21/711 (3%) were partially hatched from the zonae (Table 2). The zona-free zygotes were considered arrested and were removed from the culture.

Day 3: 4-8-cell stages.

Removal of the 34 zona-free embryos on day 2 left 677 for further evaluation. Eleven of the 677 (2%) had completely lost their zonae and 90/677 (15%) were partially extruded. On day 3, 586/677 (87%) of oocytes fertilized by zona drilling cleaved to 4 cells. Within the partially extruded group were embryos in which blastomeres linerally extended out through the zona (Fig. 2a). In addition, in a few cases, extrusion out of the zona resulted in complete loss of a blastomere (Fig. 2b). These patterns contrasted with the normal tetrahedral structure of the 4-celled embryo (Fig. 2c).

Day 4: morula stage.

On the morning of Day 4, 485/586 (83%) of embryos which had cleaved to 4 cells continued development to the morula stage, and 306/372 (82%) of the control oocytes were compacting. Within the manipulated group the compacted embryos were subdivided into zona-free (17/485, 4%), partially extruded (36/485, 7%), and normal categories. A frequent observation was that partial extrusion of the embryo on previous days was associated with exclusion of the extruded blastomeres from the zygote proper at the compaction stage (Fig. 3). Partial extrusion at the morula stage itself was not associated with blastomere loss

(fig. 4).

Day 5: blastulation.

Of morulae in the zona-drilled group, 66% (321/485) reached the blastocyst stage, while 59% (181/306) of the control embryos became blastocysts. While this difference was not statistically significant (Table 3), there was a significant increase in the percentage of inseminated oocytes which reached the blastocyst stage (Table 1). This is due to the higher rate of fertilization in the zona-drilled group. Morphological variants peculiar to zona-drilled embryos were again seen. In 6 of 321 instances (2%), two separate blastocoels, one inside and one outside the zona were observed (Fig. 5). In addition, many of the embryos that had prematurely lost their zonae at the 4-cell or morula stages were observed to have aggregated to form giant blastocysts. This occurred despite the large culture volume (2 ml) and the fact that relatively few embryos lost their zonae prematurely.

Attrition rate during cleavage.

Table 3 summarizes losses at each stage of cleavage of zona-drilled and control embryos. These numbers were collected beginning on day 2, when zona-drilled embryos which completely lost their zonae prior to the 2-cell stage had been removed. On each culture day, the percentage of normal embryos from the preceding day which cleaved was calculated. These data thus determine if zona-drilled embryos are prone to arrest at a specific stage of cleavage.

As shown, the percentages of control and experimental embryos which failed to develop on each culture day were approximately the same. The

data show that after fertilization by zona drilling, embryos at all stages of preimplantation development are normally viable. The number of zona-drilled embryos which reached the blastocyst from the morula stage was slightly greater in the zona-drilled group. However, this difference was not statistically significant (Table 3).

DISCUSSION

In the present experiments, oocytes subjected to zona drilling were studied in vitro from the time of insemination through cleavage to the blastocyst stage, and compared with zona intact controls. As reported previously (Gordon and Talansky, 1986), zona drilling was associated with a significant increase in the fertilization rate. A corresponding increase in the percentage of inseminated ova which reached the blastocyst stage was seen in this study (Table 1). As with in vivo experiments previously conducted, the results of in vitro culture reported here indicate that zona drilling does not lead to fertilization of biologically abnormal eggs which are unable to develop. Rather, zona drilling of a cohort of eggs results in a greater number of normally developed blastocysts than a comparable group of oocytes not subjected to manipulation. Evaluation of cleavage in vitro has further shown that the rate of attrition of zona-drilled embryos is not different from controls at any stage of cleavage development (Table 3). Thus, after fertilization, the in vitro cleavage survival of zona-drilled eggs is indistinguishable from normal controls.

Characteristics of fertilization and early development unique to

zona-drilled eggs were observed at all stages. On the day of insemination, preferential binding of sperm to the exposed membrane of zona-drilled oocytes was observed, and pronuclei consistently formed earlier in zona-drilled eggs. During subsequent cleavage, zygotes became partially or totally extruded from the zona, and these events were associated with blastomere loss and the appearance of small, twin blastocysts. Some aggregation of prematurely "hatched" embryos also occurred.

The "clustering" of spermatozoa at the exposed portion of the oocyte membrane (Fig. 1) is probably indicative of normal processes of oocyte activation and development of the block to polyspermy at the zona pellucida. In the mouse, the zona block is accompanied by reduced sperm binding (Sato, 1979), and previous studies involving direct observation of fertilization or experimental oocyte activation have shown that the zona block develops within minutes of oocyte activation (Sato, 1979; Gwatkin et al., 1973). When oocytes are subjected to zona drilling, contact between spermatozoa and the egg occurs almost immediately after insemination. Consequent activation of the egg induces the zona block, a very few sperm are then able to bind to the zona. The importance of this observation is that introduction of a large gap in the zona does not appear to result in diffusion of the cortical granule contents out of the perivitelline space with resultant loss of the the ability to induce the zona block.

More rapid appearance of pronuclei in zona-drilled eggs undoubtedly results from elimination of the time normally required for zona penetration. It is possible that this feature of fertilization after zona drilling might actually improve the chance of obtaining a human

pregnancy. There is a positive correlation between more rapid cleavage development of human eggs and the likelihood of conception (Trounson et al., 1982; Mohr et al., 1983). If reduction of the time between insemination and pronucleus formation in human eggs were to lead to more advanced cleavage development during the culture period, the result could be an increased probability of implantation. We consider this possibility unlikely for two reasons. First, precocious fertilization of zona-drilled eggs was not associated with a significantly increased cleavage rate. Second, in human cases, the correlation between rapidity of cleavage and implantation is likely a reflection of overall health and developmental vigor of the zygote, characteristics not likely to be affected by reducing the time required for zona penetration.

It is unclear whether partial extrusion of zona-drilled embryos leading to loss of blastomeres during early cleavage (Fig. 2a,b) reduces embryo viability. Separation of blastomeres at the 2- or 4-cell stage does not kill the embryo, and might even lead to development of multiple genetically identical concepti (Tarkowski, 1959; Tarkowski and Wroblewska, 1967). At later stages, experimental removal of a blastomere for genetic diagnosis or other purposes does not interfere with development of the biopsied morula or blastocyst (Gardner and Edwards, 1968; Monk et al., 1987). These data indicate that loss of single blastomeres as observed in our experiments would not necessarily preclude embryo survival. However, dissociation of 8-celled embryos leads to inability of the isolated blastomeres to produce inner cell mass cells at the blastocyst stage, and consequently, the implantation rate is severely reduced (Tarkowski,

1967). This finding does raise the prospect that loss of several blastomeres from zona-drilled embryos at the 8-cell stage might lead to embryo death. While we did not observe such multiple blastomere separation, we cannot rule out the possibility that after zona drilling, embryos might occasionally be lost by this mechanism.

The formation of independent blastocysts from portions of the embryo inside and outside the zona (Fig. 5) could increase the possibility of twinning. Bisection of 2-cell, 4-cell or morula stage embryos can lead to development of two small blastocysts which, after transfer to the oviducts, develop into identical twins (Willadsen, 1979). While twinning must obviously be distinguished from embryo loss, it is of significance to human IVF. If several embryos are to be transferred in a clinical IVF procedure, and if one or more "double blastocysts" is among them, an undesirable increased risk of multiple gestation may be incurred.

Spontaneous aggregation of zona-drilled embryos is also of potential importance. Although the ability of zona-free embryos to aggregate in vivo has not to our knowledge been tested, in vitro aggregation followed by embryo transfer readily leads to birth of adult genetically mosaic mice (Mintz, 1965) rats (Mayer et al., 1974) rabbits (Gardner and Munro, 1974) and even sheep or goats (Tucker et al., 1974). Indirect evidence also exists for spontaneous in vivo aggregation of human embryos (Corey et al., 1967; de la Chapelle et al., 1974).

These previous results, as well as our observation that zona-drilled embryos may lose their zonae and aggregate spontaneously in culture, make it important to determine if transfer of several

zona-drilled embryos into a single recipient could be lead to development of aggregation chimeras. We are currently conducting such experiments in mice using genetic markers which will maximize the probability of detecting chimerism. If in vivo aggregation is found, we will conclude that transfer of multiple zona-drilled embryos into a single individual is unadvisable, and that in circumstances where multiple zygotes are available for transfer, freezing of supernumerary embryos should be undertaken.

We are also determining whether modification of the zona drilling technique might prevent any of the potential problems described here. As in our initial experiments (Gordon and Talansky, 1986) the hole introduced into the zona in the present study was large, and frequently exposed substantial portions of the oocyte surface. Such a protocol, while markedly improving the efficiency of fertilization, also renders the embryo more prone to premature loss of the zona. We have initiated experiments in which the hole introduced into the zona is exceedingly small. In this unaggressive approach, we terminate the drilling process immediately upon penetration of the zona, and the hole produced is not visible when the procedure is completed. We have thus far drilled approximately 50 eggs in this manner and have observed none of the cleavage abnormalities described in this report. Whether such small holes will also lead to improved fertilization rates when sperm counts are low is under investigation.

ACKNOWLEDGEMENTS.

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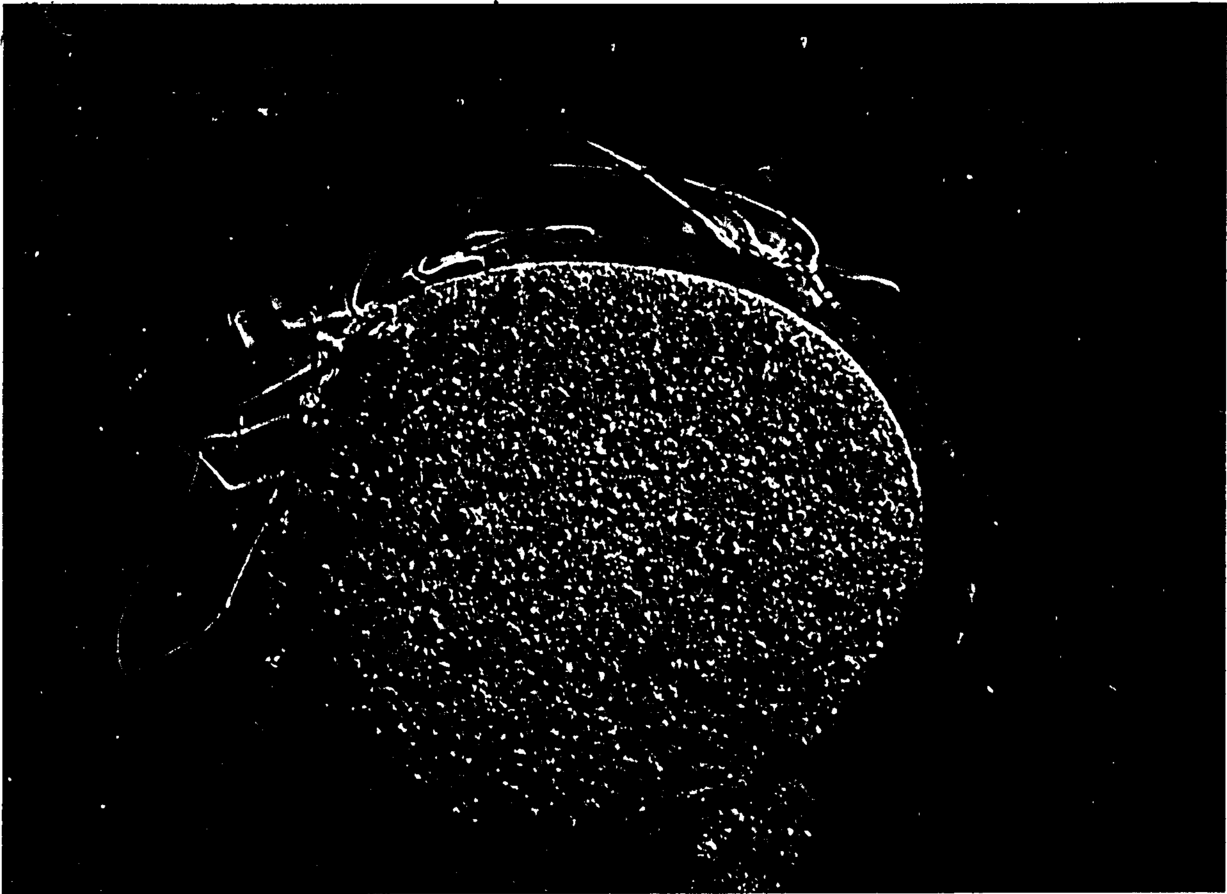
LEGEND FOR FIGURE 1

Figure 1. Mouse oocyte 35 min post insemination. Sperm are concentrated at the area of the plasma membrane exposed by zona drilling. x800.

FIGURE 1



LEGEND FOR FIGURE 2

Figure 2a. Zona-drilled embryo at the 4-cell stage of development. The blastomeres are linearly extending out through the hole created by drilling. 2b. A zona-drilled embryo at the 4-cell stage showing linear cleavage and loss of blastomere. 2c. A control 4-celled embryo from the same culture day, showing the normal tetrahedral structure. x150.

FIGURE 2a

FIGURE 2b

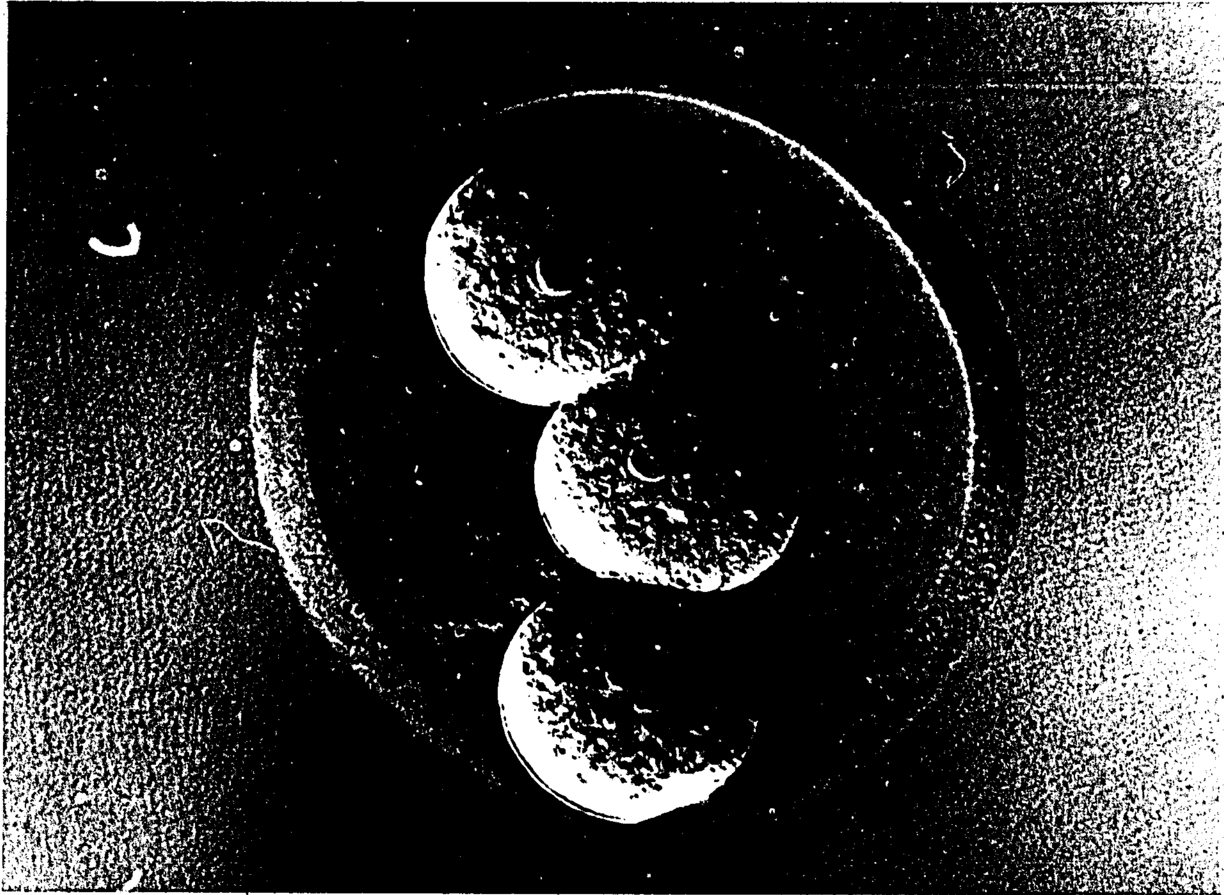
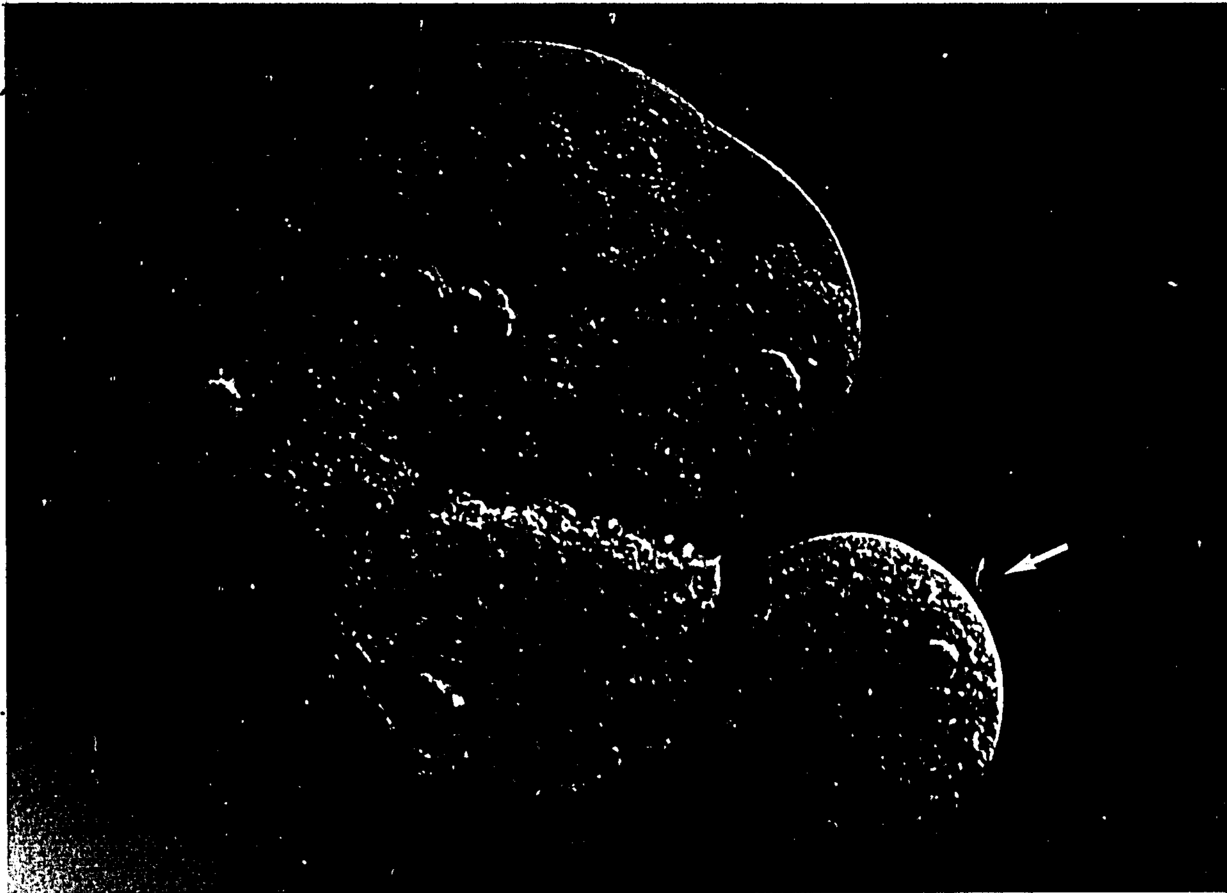


FIGURE 2c

LEGEND FOR FIGURE 3

Figure 3. Zona-drilled embryo at the late 4-cell-early morula stage. Arrow indicates where a single cell extruded through the zona before compaction began. The zona was lost during preparation. x200.

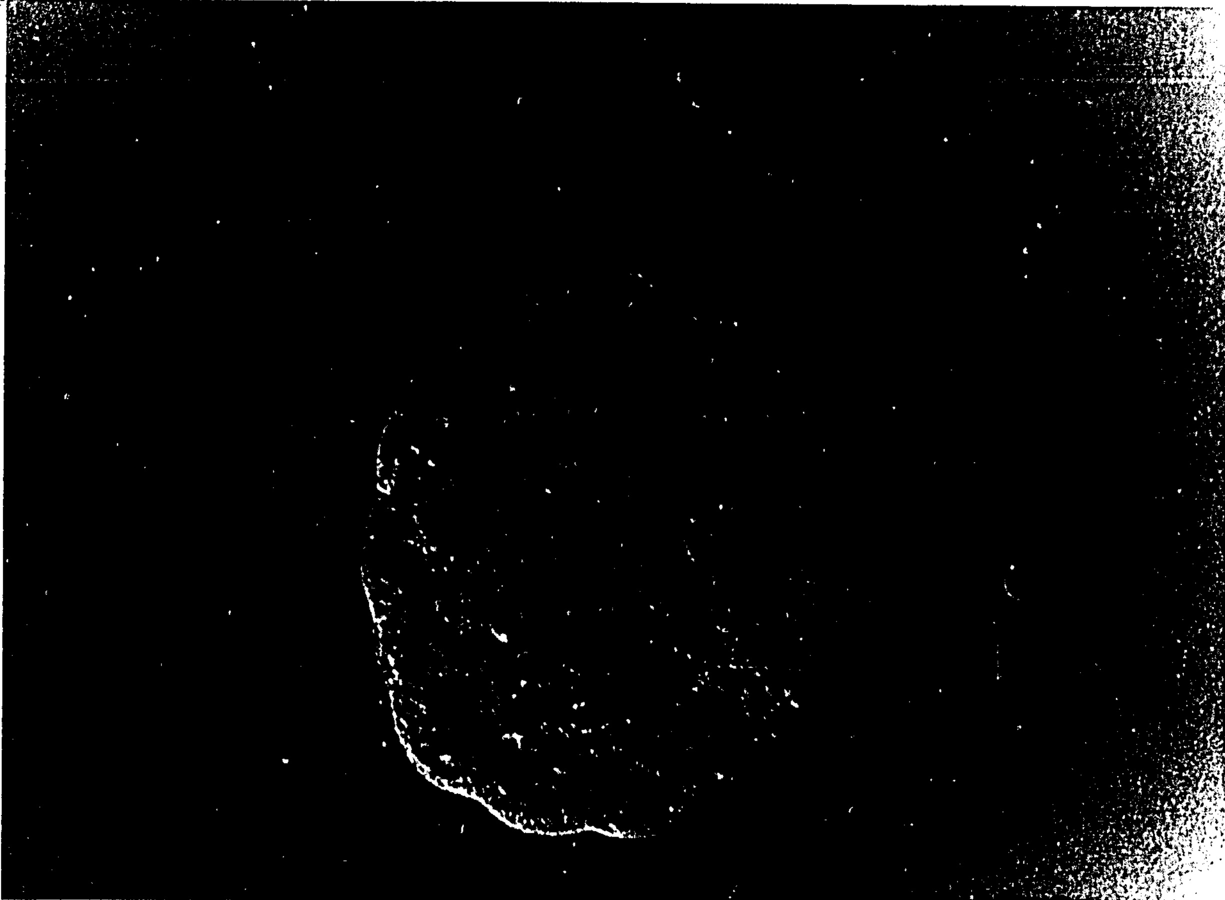
FIGURE 3



LEGEND FOR FIGURE 4

Figure 4. Zona-drilled morula at the compaction stage. The extrusion through the zona occurred after compaction began with no subsequent loss of cytoplasm. x200.

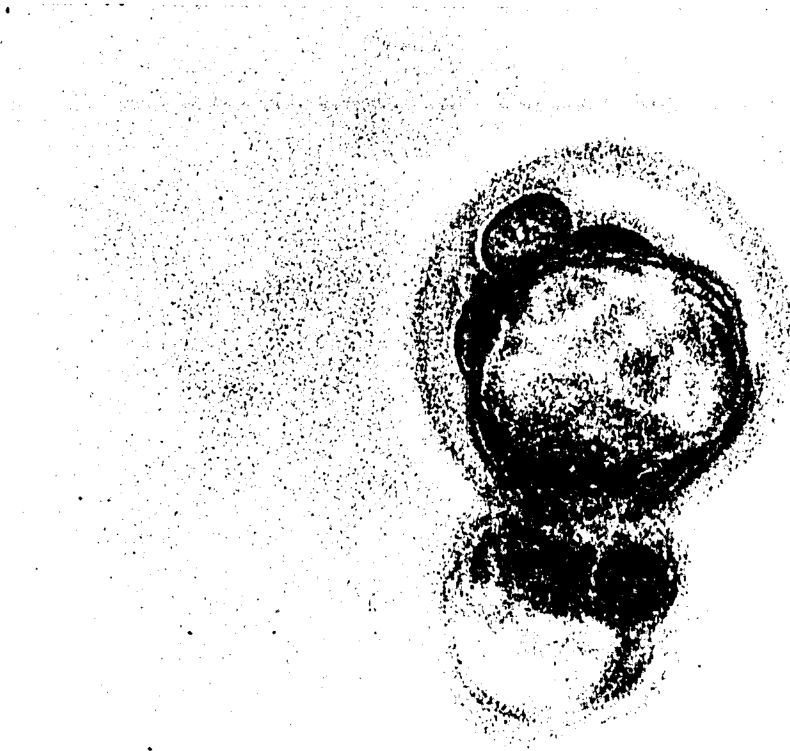
FIGURE 4



LEGEND FOR FIGURE 5

Figure 5. Formation of twin blastocysts resulting from zona drilling. One blastocyst is outside the zona, the other inside. x100.

FIGURE 5



LEGEND FOR TABLE 1.

Table 1. The numbers of inseminated and fertilized zona-drilled ova which developed to the blastocyst stage in comparison with controls.

TABLE 1. Fertilization and development of zona-drilled eggs.

	FERT./INSEM.	(%)	BLAST./INSEM.	(%)	BLAST./FERT.	(%)
Control	440/847	(52)	181/847	(21)	181/440	(41)
Drilled	711/913	(78)[a]	321/913	(35)[a]	321/711	(45)[b]

a: $p << .001$

b: $.1 < p < .25$

LEGEND FOR TABLE 2

Table 2. Cleavage abnormalities of zona-drilled oocytes. Dashed lines indicate that it was not posssible to evaluate abnormalities at the designated stages.

TABLE 2. Cleavage abnormalities of zona-drilled oocytes.

	STAGE EVALUATED			
	2-CELL	4-CELL	MORULA *	BLASTOCYST
Loss of zona	34/711	11/677	17/485	---
Partial extrusion	21/711	90/677	36/485	---
Twinning	----	----	----	6/321

* Only morulae undergoing compaction at the time of observation were recorded.

LEGEND FOR TABLE 3

Table 3. Embryos which had reached the 2-cell stage and had not lost the zona were evaluated for subsequent development. At each stage of cleavage, the percentage of normal embryos on each culture day which cleaved further is noted. Thus on every culture day, the numerator of each fraction is the same as the denominator on the previous day. The number of morulae which developed to the blastocyst stage was slightly higher in the zona-drilled group; however, this difference was not statistically significant.

TABLE 3. Attrition rates of zona-drilled embryos during cleavage.

	ZONA-DRILLED	(%)	CONTROLS	(%)
2-CELL	677		440	
4-CELL	586/677	(87)	372/440	(85)
MORULA	485/586	(83)	306/372	(82)
BLASTOCYST	321/485	(66)[a]	181/306	(59)

a: $0.025<p<0.05$

DISCUSSION

Infertility cannot be effectively treated unless we gain a thorough understanding of the intricate process of fertilization. In vitro fertilization (IVF) has provided not only a therapeutic approach to infertility, but also an invaluable research tool with which to investigate gamete interactions. In the context of IVF, micromanipulation has furthered our ability to understand the events of fertilization. The experiments described in this thesis research represent a series of studies designed to increase our understanding of the use of gamete micromanipulation to improve IVF. A discussion is included at the end of each chapter in which the results and data of the particular study are interpreted. The present discussion serves to combine the findings from all four chapters and suggest prospects for future research.

In Chapter I, we used the murine model in an attempt to develop a method of fertilization by subzonal sperm microinjection. While we failed to achieve fertilization, some intriguing observations prompted us to more closely examine the physiology of the fertilizing sperm. Specifically, we noted that manipulation caused sperm to be immobilized. The potential role for sperm motility in sperm-egg fusion was investigated in Chapter II. In these studies, we used biochemical manipulation to address the notion that immobilization of sperm was

altering the membrane ion potential and interfering with gamete fusion.

Studies in Chapter II indeed suggest that the sperm must maintain a specialized membrane status for the retention of fertilizing competency. In these experiments, sperm were exposed to specific inhibitors of the Na^+,K^+-ATPase. Treated sperm were first acrosome-reacted to bypass the inhibitory effect of ouabain on this preliminary but required event. Since sperm exposed to ouabain were unable to fertilize zona-free oocytes, we inferred that acrosome-reacted sperm may require a functional Na^+,K^+-ATPase in order to maintain the ability to fertilize an oocyte (Talansky et al., 1987). Although our results favor the existence of ion pump function on the sperm membrane, we were not able to entirely dissociate possible effects of ouabain on the oocyte. Our methodology could be improved with the application of an irreversible inhibitor. This would rule out any chance of simultaneous exposure to both gametes, and its effects on sperm or oocytes upon subsequent fertilization could be assessed independently. If these experiments positively demonstrate a role for functional Na^+,K^+-ATPases on the sperm membrane, it may be possible to reverse altered potentials on the gamete membrane which occur during sperm injection. Using an electroporation device one can transiently change the distribution of ions. This method has previously been used to facilitate transfer of DNA into cells (Potter et al., 1984). Since its effect is based on the alteration of membrane potential, electroporation might be used to overcome perturbations of ionic distributions induced by manipulations. This technique might therefore improve the mouse system.

The fact that our microinjection procedure was interfering with the sperm fertilizing capacity led us to explore alternative methods of micromanipulation to assist IVF. To do this, we approached fertilization by manipulating the oocyte. Using a micromanipulator, we created small holes in the outer zona pellucida of the oocyte thereby allowing sperm direct access to the oocyte membrane. After this "zona drilling" procedure, the egg was inseminated as in a routine IVF procedure. Therefore, zona drilling enabled us to establish fertilization while preserving intact sperm function and motility. However, results from the application of this technique, described in Chapter III, raised questions about the role of the zona in maintaining the integrity of the preimplantation embryo. We were particularly interested in the repercussions of local zona dissolution on early cleavage. Thus, studies of the developmental patterns of oocytes inseminated and fertilized after zona drilling were conducted, and results are presented in Chapter IV.

Prior to zona drilling, two different approaches directed at achieving fertilization by micromanipulation, microinjection and subzonal insertion, have been studied. It is agreed that before the clinical application of such methods, a test model should be established which addresses two essential issues. First it is important to determine if a single sperm can fertilize the oocyte after microinjection. Second, we must ask if an oocyte fertilized by this technique has the potential to develop into a viable individual. Microinjection of sperm or sperm heads directly into the ooplasm has been attempted in both heterospecific (Thadani, 1980) and homospecific (Uehara and Yanagimachi, 1976; Markert, 1983; Barg et al., 1986)

systems. Though these microinjections occasionally resulted in formation of male pronuclei (Uehara and Yanagimachi, 1976; Thadani, 1980; Markert, 1983; Barg et al., 1986), death of the oocyte was frequent, especially in the experiments where mouse sperm were directly injected into oocytes of the same species (Markert, 1983; Barg et al., 1986). A second approach to assisted fertilization has been sperm injection into the perivitelline space. As before, these methods have been applied to both heterospecific (LaSalle et al., 1987) and homospecific (Barg et al., 1986; LaSalle et al., 1987; Laws-King et al., 1987) gametes. Subzonal insertion of several human sperm into hamster oocytes led to the formation of swollen sperm heads (LaSalle et al., 1987). Although head decondensation is probably a good indication of the potential for the male gamete to form a pronucleus, it does not necessarily represent true fertilization and does not differ from what is observed using the standard hamster penetration assay. Thus, it would be desirable to refine the microinjection techniques in a homospecific system which can manifest physiological signs of fertilization, and lead to normal embryo cleavage.

These criteria have been satisfied in experiments using human gametes (Laws-King et al., 1987; LaSalle et al., 1987). In these reports, sperm microinjections under the zona pellucida resulted in fertilization and embryo cleavage. Single sperm injections carried out by Laws-King et al. (1987) were followed by the formation of pronuclei, release of second polar bodies, and the loss of cortical granules. Further, the authors ruled out parthenogenetic activation by electron microscopy and karyotypic analysis. These results, especially the indications of fertilization, are more physiologically sound than the

appearance of swollen sperm heads from heterospecific experiments. However, as mentioned earlier, a major goal of gamete micromanipulation is the production of viable offspring. Since it would be inappropriate to establish an experimental protocol in the human species, we must utilize a homospecific animal model to test sperm microinjection and embryo transfer. In this regard, the mouse has proven highly problematic. Both we and others have obtained poor results using mouse gametes for in vitro micromanipulation. It is not clear what distinguishes the success in the human system from the microinjections attempted with mouse gametes. Due to as yet undefined interspecies differences, the mouse seems an unsuitable model with which to pursue micromanipulation. Therefore, other homospecific animal models should be investigated.

Hamster sperm microinjection might be an appropriate animal model for this procedure. Previous attempts to achieve hamster egg fertilization by microinjection have been promising (Uehara and Yanagimachi, 1976). Because the hamster egg does not present any significant block to polyspermy at the level of the plasma membrane (Barros and Yanagimachi, 1972; Binor et al., 1982), it more closely resembles the human oocyte (Gordon et al., 1988) than mouse eggs which have a partial plasma membrane block (Braden et al., 1954; Wolf, 1978). Until recently, the ability to utilize the hamster as an animal model for in vitro micromanipulation was hampered by failure of the embryo to develop beyond the first cleavage division in vitro. This two-cell block has recently been overcome by reducing the volume of culture medium (Schini and Bavister, 1988). This protocol, in conjunction with improved gamete preparation as described by Laws-King

et al. (1987) for single sperm microinjections, might result in successful fertilization and cleavage of hamster eggs fertilized by microinjection, and thereby allow an assessment of developmental potential of such zygotes.

Results of studies in Chapters III and IV on assisted fertilization by zona drilling establish that minor manipulations of the zona pellucida can lead to successful fertilization even at drastically reduced sperm concentrations. In addition, other work from our laboratory demonstrates that zona drilling can rescue the fertility of a line of infertile male mice whose sperm are incapable of penetrating intact zonae (Gordon, 1988). Findings such as these, in conjunction with our report that zona drilling leads to low rates of polyspermy and the birth of normal live young following embryo transfer (Gordon and Talansky, 1986), all suggest that this technique could have potential use in human IVF. Unlike subzonal insertion and sperm microinjection, in which sperm are subjected to direct contact with a microneedle, zona drilling involves standard insemination and thus requires that sperm be motile. However, in both our mouse studies and our initial human studies, we have also attained fertilization with submotile populations of sperm. Therefore, results obtained thus far, indicate that the most appropriate clinical application of zona drilling would be in cases of infertility where sperm are incapable of traversing intact zonae, or in instances where oocytes lack a zona pellucida receptor required for sperm binding and penetration.

Our zona drilling studies have not yet addressed the possibilty that completely immobilized sperm can fertilize drilled oocytes. Could such sperm actually "fall through the hole" and proceed to fuse with

the vitelline membrane? This would be a difficult theory to test in the mouse since our previous attempts to fertilize oöcytes with immobilized sperm failed (Barg et al., 1986). However, results from human:hamster experiments (Aitken et al., 1983) suggest that the hamster oocyte might be a suitable animal model with which to study this problem. In that study, immotile sperm from Kartagener's Syndrome patients successfully penetrated zona-free hamster oocytes. Therefore, application of zona drilling to the hamster system might be helpful in evaluating the usefulness of this technique in cases of infertility due to complete sperm immotility. As previously stated, a homospecific test system would be desirable. Advances in hamster embryo culture alluded to previously might make such assessments possible.

Studies in Chapter IV describing the in vitro development of oocytes fertilized after zona drilling provide further insight into possible risks associated with the clinical application of gamete micromanipulation. Abnormal cleavage and aggregation of drilled embryos occurred at relatively low frequencies (Talansky and Gordon, 1988). Furthermore, _in vitro_ aggregation of embryos to form genetic chimeras requires aggressive manipulation and does not readily occur spontaneously (Mintz et al., 1973). For these reasons, we feel that the chances are low that such problems will occur spontaneously _in vivo_ after transfer of manipulated embryos. However, in refining zona drilling for clinical use, measures should be taken to create holes of adequate proportions to allow for increased rates of fertilization, but limited enough to maintain the overall integrity of the zona in order to eliminate any risk of oocyte extrusion. We have begun to explore the use of "unaggressive" drilling in order to address this issue.

Intrusion on the integrity of the zona barrier before cleavage proceeds has lead to some intriguing fundamental questions and prospects for future research. These issues, addressed below, might also eventually be studied in the human system:

1) What are some potential ramifications of zona disruption on later preimplantation development?

In the rabbit, the presence of an intact zona is required for implantation (Kane, 1972). However, in other species such as the mouse, it has been shown that zona-free embryos are capable of fertilization and give rise to normal offspring after embryo transfer (Thadani, 1982). Our results in Chapter IV suggest that zona drilling, which results in an oocyte in a condition intermediate between zona-free and zona-intact, can result in cleavage distortions which may have detrimental effects on later development. Partial violation of the zona may be more harmful than total removal of the zona before the initiation of cleavage. In the latter case, the blastomeres may have the opportunity to establish an association with each other. In contrast, zona drilling, by creating a path of low resistance, could induce an uneven "leakage" or extrusion of blastomeres during their initial period of association.

Relevant to this issue, it has been shown that in mouse embryos early junctional communication among blastomeres is established at the late 8-cell stage (early compaction) (Lo and Gilula, 1979). Lee et al. (1987) have recently demonstrated that this gap junctional communication is involved in the process of embryo compaction. The importance of gap junctions and tight junctions in embryonic

development has been suggested by the fact that cellular determination occurs concommitantly with the onset of gap junctional communication (Gardner and Rossant, 1976). It has additionally been theorized that factors distinguishing cells destined to become trophoblast and inner cell mass might include the formation of "gradients" established by the gap junctions (Lo and Gilula, 1979). Furthermore, these investigators have discussed the role of tight junctions in generating permeability barriers which create distinct microenvironments important for the differentiation of the early embryo. It is possible that separation of blastomeres at earlier stages of association may alter formation of gap junctions and tight junctions. Thus normal development, including the organization of the early embryo, may be hindered by significant disruption of the zona.

The role of the zona in organizing the human embryo during cleavage and implantation has not been studied. It would therefore be of interest to conduct ultrastructural studies on human oocytes which are considered unsuitable for embryo transfer after zona drilling. Examination of these manipulated oocytes or embryos for the status of gap junctions and tight junctions could provide information about the role of an intact zona in the establishment of these junctional complexes in the human.

2) Does zona drilling affect hatching of the blastocyst?

Perona and Wassarman (1986) have localized a proteinase termed" strypsin" in the mural trophectoderm of mouse embryos which is directly involved in hatching blastocysts from their zonae. This enzyme is inhibited by a variety of agents including soybean trypsin inhibitor.

If drilled embryos are exposed to such inhibitors prior to blastocyst formation, they may then be evaluated for their ability to hatch *in vitro*. Failure of such embryos to hatch may indicate that the portion of zona located nearest the mural trophectoderm is the only area from which hatching can occur. In contradistinction, successful hatching of drilled embryos following exposure to soybean trypsin inhibitor would indicate that hatching need not occur near the mural trophectoderm for normal implantation and development. Thus a failure in the ability of an otherwise normal embryo to hatch from its zona could be potentially corrected by the zona drilling procedure.

In conclusion, the data presented in this dissertation illuminate and clarify some of the technical and biological hurdles which must be overcome to further understand and improve mammalian reproduction. In vitro fertilization circumvents the obstacles encountered by the male gamete in the female reproductive tract prior to immediate contact with the oocyte. However inefficient at its present level of sophistication, this method has contributed significantly to our understanding of gamete interaction and to the ability to treat human infertility. Our grasp of conception has been further improved by the recent development of gamete micromanipulation. These methods can be used to bypass the final barrier to sperm penetration and may ultimately enable IVF to be accomplished with a single spermatozoon. This achievement will not only deepen our understanding of the fertilization process, but will also allow the reproductive biologist to control that process so as to improve the reproductive health and efficiency of animals and human beings.

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