INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

ACTIVATION OF BOVINE OOCYTES FOLLOWING INTRACYTOPLASMIC SPERM INJECTION (ICSI)

by

Jin-Tae Chung

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

Department of Animal Science
Macdonald Campus McGill University
Montreal, Quebec, Canada

Suggested short title:

Intracytoplasmic sperm injection of bovine oocytes

ACTIVATION OF BOVINE OOCYTES FOLLOWING INTRACYTOPLASMIC SPERM INJECTION (ICSI)

ABSTRACT

M.Sc.

Jin-Tae Chung
Animal Science

The objective of this study was to develop a reliable method for intracytoplasmic sperm injection (ICSI) in bovine oocytes. Oocytes recovered from abattoir-derived ovaries were centrifuged for 5 min at 6000×g to facilitate sperm injection. Sperm were pre-treated *in vitro* with 5mM dithiothreitol (DTT), and diluted (approximately 1:5) with 5% polyvinylpyrrolidone (PVP) in 0.9% saline. After sperm injection, various activation procedures were compared. Initially, 3 h after activation with 5μM Ionomycin (A23187), oocytes with second polar bodies were selected and treated with 1.9mM 6-dimethylaminopurine (DMAP). The cleavage rate of sperm-injected oocytes treated with Ionomycin and DMAP was higher than with Ionomycin alone (62.1 vs. 27.3%, $p<0.05$). In sham-injected control oocytes treated with Ionomycin and DMAP, the cleavage rate was approximately six times higher than that of oocytes treated with Ionomycin alone (44.3 vs. 7.4%, $p<0.001$). Upon examination 16 h after ICSI, pronuclear formation was observed in 33 of 47 (70.2%) DMAP-treated oocytes. Two pronuclei were present in 18 of 33 (54.6%), while one and three pronuclei were seen in 8 of 33 (24.2%) and 7 of 33 (21.2%), respectively. In sham-injected DMAP-treated control oocytes, 6 of 15 (40.0%) had one pronucleus while 9 of 15 (60.0%) had two pronuclei. Since a single Ca^{2+} stimulation provided insufficient activation and DMAP treatment could result in triploidy, activation by multiple Ca^{2+} stimulations following ICSI was tested. Three Ca^{2+}

stimulations were given at 30-min intervals. Pronuclear formation was observed in 16 of 41 (39.0%) oocytes at 16 h after sperm injection, with one and two pronuclei found in 4 of 16 (25.0%) and 12 of 16 (75.0%), respectively. Although one pronucleus was formed in 3 of 33 (9.1%) sham-injected control oocytes treated with three Ca^{2+} stimulations, two pronuclei were not seen in any of these oocytes. Due to the low rate of pronuclear formation after 5μM Ionomycin, 50μM Ionomycin was compared in the same multiple treatment protocol. The higher dose (50μM) resulted in a higher incidence of pronuclear formation (40.9 vs. 26.3%) after ICSI. The rate of metaphase III (MIII) arrest was lower and the rate of pronuclear formation was higher in sperm injected oocytes suggesting that the sperm did contribute to the activation process. Further improvements are required in order to increase the rate of normal activation following ICSI in the bovine oocyte.

ACTIVATION D'OVOCYTES BOVINS SUIVANT L'INJECTION INTRACYTOPLASMIQUE D'UN SPERMATOZOIDE (ICSI)

RÉSUMÉ

L'objectif de cette étude était de développer une méthode fiable pour les injections intracytoplasmiques de sperme (ICSI) chez les ovocytes bovins. Des ovocytes réchappés d'ovaires provenant d'un abattoir ont été centrifugés pendant 5 min. à 6000x*g* afin de faciliter l'injection de sperme. Le sperme a été prétraité *in vitro* à l'aide de 5mM de dithiothreitol (DTT), et dilué (environ 1:5) avec 0.9% de solution saline contenant 5% de polyvinylpyrrolidone (PVP). Plusieurs procédures d'activation ont été comparées suivant l'injection de sperme. Trois heures après l'activation avec 5μM d'Ionomycin (A23187), les ovocytes ayant globule polaire secondaire ont été sélectionnés et traités à l'aide de 1.9mM 6-dimethylaminopurine (DMAP). La vitesse de division des ovocytes injectés de sperme et traités à l'Ionomycin et au DMAP était supérieure à celle des ovocytes traités à l'Ionomycin seulement (62.1 vs 27.3%, $p<0.05$). Chez les ovocytes témoins (injectés sans sperme) traités à l'Ionomycin et au DMAP, la vitesse de division était approximativement six fois plus haute que pour les ovocytes traités avec l'Ionomycin seul (44.3 vs 7.4%, $p<0.001$). Lors de l'observation, 16 heures suivant l'ICSI, une formation pronucléaire pouvait être constatée chez 33 des 47 (70.2%) ovocytes traités au DMAP. Deux pronuclei étaient présents dans 18 des 33 (54.6%) ovocytes, tandis que un et trois pronuclei ont été observés dans 8 des 33 (24.2%) et 7 des 33 (21.2%) ovocytes, respectivement. Chez les ovocytes témoins traités au DMAP et injectés avec la solution PVP sans sperme, 6 des 15 (40%) avaient un pronucleus tandis

que 9 des 15 (60%) avaient deux pronuclei. Puisqu'une simple stimulation à l'aide de Ca^{2+} était insuffisante pour activer les ovocytes et que le traitement au DMAP pouvait mener à trois pronuclei, l'activation par de multiples stimulations de Ca^{2+} suivant l'ICSI a été testée. Trois stimulations de Ca^{2+} ont été données (intervalles de 30 min). Une formation pronucléaire a été observée dans 16 des 41 (39.0%) ovocytes, 16 heures suivant l'injection de sperme, avec un et deux pronuclei dans 4 des 16 (25.0%) et 12 des 16 (75.0%) ovocytes, respectivement. Même si un pronucleus a été formé dans 3 des 33 (9.1%) ovocytes témoins injectés de la solution PVP et stimulés à trois reprises avec du Ca^{2+}, aucun des ovocytes ne contenait deux pronuclei. Étant donné le faible taux de formation pronucléaire après 5μM d'Ionomycin, les effets de 50μM d'Ionomycin ont été étudiés. La dose élevée (50μM) a mené à une plus grande incidence de formation pronucléaire (40.9 vs 26.3%) suivant l'ICSI. L'arrêt de la métaphase III était plus courte et la formation pronucléaire était plus élevée chez les ovocytes injectés de sperme. Ceci démontre que le sperme a contribué au processus d'activation. Des améliorations supplémentaires sont requises afin d'augmenter le niveau d'activation normale suivant l'ICSI chez les ovocytes bovins.

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I owe my thanks to many people who have provided invaluable help in the preparation of this thesis. I am grateful to the members of my committee, Dr. Bruce R. Downey, Dr. Carol L. Keefer and Dr. Daniel Bousquet for their supervision, encouragement, technical advice and assistance in my research.

I would also like to thank to Sophie Poulin, Heather L. Watt, Jan Pika and Denise Laurin for their valuable assistance and co-operation.

I would especially like to thank to Boviteq Inc. and Nexia Biotechnologies Inc. for their support and co-operation.

Most of all, I truly express appreciation to my parents, my wife, Hye Kyeong and my children, Jonah and Jenny for their endless love and support.

LIST OF FIGURES

LIST OF TABLES

1. GENERAL INTRODUCTION

In the dairy industry, artificial insemination (AI), *in vitro* fertilization (IVF) and embryo transfer (ET) have rapidly developed into important biotechnological tools in farm animal reproduction. Artificial insemination is largely responsible for substantial rates of improvement for milk yield through the facilitation of progeny testing and genetic improvement programs. Embryo transfer has provided the means to exploit superior genes on the female side. *In vitro* production permits the use of immature oocytes from genetically superior females. However, there are limiting factors in the efficiency of *in vitro* embryo production such as sire variability and male infertility. Therefore, introduction of technologies like the injection of sperm into the oocyte could provide a solution to several male related problems.

Intracytoplasmic sperm injection (ICSI), one of the methods of assisted fertilization, is the mechanical placement of a spermatozoon directly into the cytoplasm of an oocyte. With the use of this technique, the normal barriers to sperm penetration including the cumulus cell-hyaluronic acid matrix, the zona pellucida and the ooplasmic membrane are bypassed. ICSI, therefore, may aid overcoming problems associated with sperm penetration of oocyte. For example, a limiting factor in the use of IVF is the variability between sires in their ability to fertilize oocytes in an *in vitro* environment. Sperm from approximately one-third of sires in use produce high oocyte penetration rates *in vitro*. Sperm from other sires provide mediocre results (~30%), fail to penetrate the oocyte (~30%), or respond too strongly resulting in polyspermy (<5%) (Keefer, 1995). While it is felt that this variability is related to capacitation, a step that all sperm must

undergo to acquire fertilizing capacity, corrective measures have not been found. Sperm microinjection could provide a solution to the problem.

Intracytoplasmic sperm injection is well established in small laboratory animals and humans, and has met with considerable success in overcoming certain human male infertility problems (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993). In mice, eighty percent of injected oocytes survived, and approximately seventy percent developed into blastocysts of which fifty percent developed into normal offspring (Kimura and Yanagimachi, 1995b). Unlike the human and mouse, there are few reports concerning sperm injection in cattle. Although the first ICSI calves were born some years ago in Japan, the factors influencing success in this species remain ill defined. However, this result demonstrates that bovine oocytes matured *in vitro* can be fertilized by sperm microinjection, and that the resulting zygotes are capable of development to the blastocyst stage. Furthermore, the viability of the embryos obtained by sperm injection was confirmed by the birth of calves after they had been transferred to a recipient cow (Goto *et al.*, 1990). Recently, a number of investigators have tried to improve the ICSI technique in cattle. However, a problem commonly encountered in the ICSI technique is the low rate of viability of injected bovine oocytes because bovine oocytes are less responsive to the injection process than other species (Keefer *et al.* 1990b). This implies that additional activation of oocytes after sperm injection is absolutely necessary to obtain pronuclear formation and subsequent cell division in cattle.

2. LITERATURE REVIEW

2. 1. Micromanipulation techniques

Since Uehara and Yanagimachi (1976) first reported that sperm nuclei introduced microsurgically into hamster oocytes could transform into well-developed pronuclei, a number of investigators have tried to obtain live young by sperm microinjection to domestic species. Micromanipulation techniques can be broadly classified as (1) those which involve opening the zona to enable direct access of sperm to the oocyte following insemination, (2) the introduction of sperm into the perivitelline space by microinjection techniques, and (3) the injection of a spermatozoon directly into the cytoplasm of an oocyte. A technique that disrupts the zona pellucida, as developed by Gordon and Talansky (1986), involves the chemical dissolution by an acidified Tyrode's solution resulting in a hole in the zona pellucida. Despite the disruption of the integrity of the zona, polyspermy is not increased when mouse oocytes are inseminated with diluted sperm. This technique is termed zona drilling. However, zona drilling may be limited to species with a block to polyspermy at the ooplasmic membrane; otherwise, problems of polyspermy could result. The technique for microinjection of sperm into the perivitelline space of the oocyte to achieve fertilization was developed by Barg *et al.* (1986), Laws-King *et al.* (1987), Mann (1988) and Ng *et al.* (1989). Live births following transfer of ova fertilized by injection of mobile spermatozoa into the perivitelline space had been obtained in mice (Mann, 1988). Zona drilling and sperm injection into the perivitelline space can be used to extend limited quantities of valuable semen. These techniques may

enable the fertilization of oocytes with low quality sperm or, in the case of microinjection techniques, with very few sperm. However, the sperm must be capacitated and maintain some motility to be used in these techniques. Mammalian sperm injected directly into the cytoplasm of oocytes, intracytoplasmic sperm injection (ICSI), can result in decondensation of the sperm head and formation of pronuclei (Uehara and Yanagimachi, 1976; Markert, 1983; Keefer, 1989) and normal embryo development (Keefer *et al.*, 1990b; Kimura and Yanagimachi, 1995ab). The ICSI technique could be used to extend valuable semen substantially and to allow limited semen usage and alternate means (e.g. freeze-drying) of semen storage, since maintenance of motility is not required as well as only a single spermatozoon is required for each oocyte. In the past, ICSI may have been limited in its usefulness due to the high risk of damage to oocytes (Keefer, 1990a), however new methods of injection have greatly decreased this risk (Kimura and Yanagimachi, 1995ab). In cattle, further improvements of sperm pre-treatment prior to injection and the activation procedure following sperm injection are required.

2. 1. 1. Intracytoplasmic sperm injection (ICSI)

Intracytoplasmic sperm injection (ICSI) is well established in small laboratory animals and humans. ICSI has become increasingly popular in human male patients with certain infertility problems (Palermo *et al.*, 1992). ICSI could be useful for analyzing normal fertilization processes as well as in determining the roles of various sperm components in fertilization and embryo development. In routine ICSI, motile spermatozoa are selected and immobilized immediately before injection. Sperm

immobilization may be performed by pressing the tail against the bottom of the dish (Dozortesv *et al.*, 1995; Keskintepe *et al.*, 1997), by freezing without cryoprotectants (Goto *et al.*, 1990; Keefer *et al.*, 1990b), or by sonication (Keefer, 1989; Goto, 1993). This immobilization, perhaps because of damage to the sperm plasma membrane, increases the fertilization rate by ICSI (Dozortesv *et al.*, 1995). In mice, eighty percent of injected oocytes survived, and approximately seventy percent developed into blastocysts of which fifty percent developed into normal offspring (Kimura and Yanagimachi, 1995b). Kimura and Yanagimachi (1995ab) reported that the nuclei of secondary spermatocytes and round spermatids injected into mouse oocytes were also capable of participating in normal embryonic development and developing into normal offspring. In the rabbit, live offspring following injection of immobilized spermatozoa into the ooplasm have been obtained (Hosoi *et al.*, 1988). Unlike the human, mouse and rabbit, there are few reports concerning sperm injection in cattle. Although the first ICSI calves were born some years ago in Japan, the factors influencing success in this species remain ill defined. However, this result demonstrates that bovine oocytes matured *in vitro* can be fertilized by sperm microinjection, and that the resulting zygotes are capable of development to term (Goto *et al.*, 1990).

Since Westhusin *et al.* (1984) reported pronuclear formation after sperm injection into bovine oocytes, a number of investigators have tried to improve the ICSI technique in cattle. However, a problem commonly encountered in the ICSI technique is the low rate of viability of the microinjected bovine oocytes. An additional problem associated with sperm injection is the inconsistent level of sperm decondensation and subsequent pronuclear formation after microinjection. One of the limiting factors is that bovine

oocytes are less responsive to the injection process than mouse or rabbit oocytes, in that only two percent of bovine oocytes are activated after the injection. Therefore, the rate of viability after microinjection of sperm into bovine oocytes is lower than in other species and, unlike other species, it implies the need for different treatments if bovine oocytes are to survive the injection procedure. Keefer *et al.* (1990b) reported that following Ionomycin (A23187) activation, 23% to 38% of sperm-injected bovine oocytes divided to the 2 to 8 cell stages after 48 h of culture. Furthermore, this method of activation was used to produce the ICSI derived calves (Goto *et al.* 1990). Sperm treatment prior to injection can also affect the ability of oocytes to activate and direct sperm decondensation and male pronuclear formation (Keefer, 1989). Perreault *et al.* (1988) reported that bovine sperm, unlike hamster sperm, could not decondense at all in hamster oocytes unless pre-treated with dithiothreitol (DTT) before injection. Pretreatment of sperm with both DTT and sodium dodecyl sulfate (SDS) also promoted ovine sperm decondensation and pronuclear formation following ICSI (Rodriguez *et al.*, 1985). Rho *et al.* (1998) have shown, in a homologous sperm-egg system, that the rates of cleavage obtained following ICSI with control and DTT-treated bovine sperm are not significantly different, but blastocyst development is significantly higher in the treated than in the control group.

2. 1. 2. Sperm-suspension medium for injection

One of the important factors affecting the outcome of intracytoplasmic sperm injection (ICSI) is the concentration of polyvinylpyrrolidone (PVP), large molecular weight polymer that is used as a sperm retardant. PVP is effective in preventing the

spermatozoa from sticking to the inner surface of the injection pipette as well as in immobilizing the spermatozoon prior to ICSI (Uehara and Yanagimachi, 1976). Gordon and Carroll (1997) examined the effect of different PVP concentrations as a sperm retardant for ICSI in bovine oocytes matured *in vitro*, as measured by cleavage rate and early embryonic development. Although the damage rate in oocytes following ICSI was affected significantly by the level of PVP in the injection medium, there was no difference in the cleavage rates in all groups studied. The value of the sperm retardant PVP in the ICSI technique is essential for successful sperm injection.

2. 1. 3. Injection pipette

The ICSI technique entails the drawing of a very fine injection pipette, just large enough to draw in a spermatozoon head and sharp enough to perforate both the zona and the oocyte plasma membrane. Fertilization and development of bovine oocytes after ICSI is affected by the outer and inner diameter (OD and ID) of injection pipettes. The percentage of embryonic development is statistically higher when using smaller diameter injection pipettes. The additional mechanical force and increased volume of injected solution when using the larger pipette might cause damage to the oocytes and arrest development (Tocharus *et al.*, 1996).

2. 1. 4. Lipid content and centrifugation of oocytes

Unlike those of the hamster, mouse and human, bovine oocytes are dark in

appearance because of a high intracellular concentration of lipid. High-speed centrifugation readily displaces intracellular lipid allowing the germinal vesicle and/or pronuclei to be identified, because the dark appearance of the cytoplasm makes it difficult to identify intracellular structures and assess meiotic status easily (Goodrowe *et al.* 1988). Wall *et al.* (1985) first used centrifugation of oocytes to facilitate pronuclear injection in order to produce transgenic cattle and pig zygotes, and centrifugation had no detectable influence on survival of the recovered embryos to 4 days. Rho *et al.* (1998) report that centrifugation at 6,000×g for 5 min for sperm microinjection is effective, and is less rigorous than the procedures used by Wall *et al.* (1985) on bovine and pig zygotes and Nagashima *et al.* (1994) on pig zygotes. This centrifugation of bovine oocytes before ICSI makes it possible to see the spermatozoon as it is expelled from the injection pipette into the cytoplasm.

Ultrastructural studies by Iwasaki *et al.* (1990) showed the presence of many lipid droplets in trophoblast cells and the inner cell mass (ICM) which, they noted, are characteristic of bovine blastocysts produced *in vitro*. This lipid content is believed to be responsible for the clear differences in buoyant density that have been demonstrated between *in vitro* matured, fertilized and cultured and *in vivo* bovine embryos (Pollard and Leibo, 1993). This lipid, present as droplets within the cytoplasm, generally has a close spatial relationship with the smooth endoplasmic reticulum within the embryo (Hyttel and Niemann, 1990) and is thought to play a role not only in providing a source of nutrients to the cell but also in modifying the physical properties and functions of membranes. However, after high-speed centrifugation of embryos, the removal of approximately 90% of intracellular lipids from zygotes has no detrimental effect on *in*

vitro and *in vivo* survival of bovine (Diez *et al.*, 1996) and porcine embryos (Nagashima *et al.*, 1994). Transfer of these delipidated embryos can apparently result in the birth of normal piglets after the usual pregnancy period (Nagashima *et al.*, 1995). Therefore, this intracellular lipid may not be essential during on *in vitro* production of bovine and pig embryos. Furthermore, centrifugation of the oocytes merely displaces lipids temporarily and, therefore, has no long-term effect on embryo viability.

2. 2. Sperm nuclear decondensation and male pronucleus formation

The nuclei of mammalian spermatozoa are genetically inactivated and structurally stabilized by association of sperm DNA with protamines, highly basic proteins that replace somatic histones during spermiogenesis. Free sulfhydryls on the cysteine residues of protamine are oxidized during transit of spermatids through the epididymis where further biochemical changes occur in the nuclei. As a result, the nuclei of mature spermatozoa in the cauda epididymis are highly stabilized by protamine disulfide bonds (Bellvé, 1979). Disruption of these disulfide bonds is a prerequisite for decondensation of the fertilizing sperm nucleus. Decondensation is a prelude to protamine replacement by histones and subsequent reactivation of the sperm genome in the oocyte (Perreault *et al.*, 1984). During decondensation, sperm nuclei show change in morphology. Nuclear swelling begins at the equatorial region of the nucleus, and initial size increases occur while the nucleus remains phase-dark. As the nucleus continues to swell, it becomes translucent and finally transparent (Perreault *et al.*, 1988).

Sperm nuclear decondensation and male pronucleus formation appear to be

related to the oocytes. Immature oocytes, prior to breakdown of the germinal vesicle (GV), do not support decondensation of disulfide-rich sperm nuclei because GV-intact oocytes are unable to reduce sufficient sperm nuclear disulfide bonds. However, once the GV has broken down and the maternal chromosomes have condensed, oocytes become capable of decondensing the sperm nucleus shortly after the reinitiation of meiosis (Usui and Yanagimachi, 1976). In contrast, disulfide-poor sperm nuclei consistently decondense in GV-intact oocytes, but do not undergo transformation into pronuclei. In ovulated hamster oocytes, both disulfide-rich and disulfide-poor sperm nuclei consistently decondense and form pronuclei following injection (Perreault *et al.*, 1984).

There are also interspecies differences in sperm nuclear decondensation and male pronucleus formation. Perreault *et al.* (1988) showed that sperm nuclei from six different species such as hamster, mouse, rabbit, bull, human and rat could undergo decondensation and pronuclear formation after microinjection into hamster ova. Human and mouse sperm nuclei decondense within 15 to 30 min of injection, and chinchilla and hamster sperm nuclei do so within 45 to 60 min, but bull and rat sperm nuclei remain intact over this same period of time. In the rat and the bull, sperm nuclei partly decondense within 3 to 6 h, but full decondensation does not occur. These interspecies differences in decondensation rates may be related to the number and/or arrangement of nuclear disulfide bonds which, in turn, is determined by the type of protamine(s) present in the sperm nucleus. For example, bull and rat sperm nuclei contain only Type I protamine. A characteristic feature of bull sperm protamine is that each cysteinyl sulfhydryl is oxidized to form a disulfide bond; thus, Type I protamine is maximally cross-linked and would be expected to be very stable. On the other hand, Syrian hamster,

chinchilla, mouse, and human sperm nuclei contain Type II protamine, as well as (and in varying proportions with) Type I protamine. Type II protamine contains less cysteine and more histidine, and might be expected to be less efficiently cross-linked. Furthermore, human sperm contain two variants of protamine II that are the least similar to Type I protamine of all mammalian protamines examined to date. Based on these interspecies differences in protamine composition and the differences in sperm nuclear stability, species with both types of protamine are less efficiently cross-linked by disulfide bonds and, therefore, more readily decondense both *in vitro* and *in vivo* (in hamster oocytes) compared with species containing only Type I protamine.

2. 2. 1. Protamine disulfide reducing agents

With the fusion of egg and spermatozoon during mammalian fertilization, the highly compact chromatin of the sperm nucleus decondenses, sperm nuclear protamines are replaced by somatic histones, the male pronucleus forms, and DNA synthesis occurs (Longo, 1981). The mechanisms by which these events occur in the egg cytoplasm are largely unknown. However, numerous studies have demonstrated that a disulfide reducing agent is required to decondense sperm nuclei *in vitro*. Sperm nuclei of all mammalian species tested to date decondense when treated *in vitro* with disulfide reducing agents alone or with reducing agents in combination with neutral detergent, anionic detergent, or protease. Treatment of sperm nuclei *in vitro* with disulfide reducing agents, in either the presence or absence of neutral detergent, results in degradation of the sperm protamine prior to sperm nuclear decondensation. Sperm-associated proteinase is

not essential for *in vivo* sperm nuclear decondensation and pronuclear formation during fertilization (reviewed by Perreault and Zirkin, 1982).

Sodium dodecyl sulfate (SDS: an anionic surfactant) and dithiothreitol (DTT: a reagent which specifically cleaves disulfide linkages) have been used to study nuclear stabilization during maturation of mammalian spermatozoa (Calvin and Bedford, 1971). Decondensation is duplicated *in vitro* by incubating pre-ejaculatory ram, rabbit or bovine spermatozoa in 1% sodium dodecyl sulfate and 2.0mM dithiothreitol. The head area of the untreated spermatozoon remains unchanged during incubation. When the spermatozoa are treated with SDS/DTT for 30 minutes, however, nuclear decondensation is evident in all spermatozoa with the extent of decondensation varying with species (Beil and Graves, 1977). In the study of Perreault *et al.* (1988), only 8% of bull and none of rat sperm nuclei showed any signs of decondensation at 60 min after injection. However, pretreatment of these sperm with DTT to reduce protamine disulfide bonds prior to microinjection significantly enhances the likelihood of their decondensing within this time period. Rho *et al.* (1998) have shown that the proportion of fertilized oocytes 16 h after ICSI is approximately four times higher with DTT-treated than with untreated sperm in bovine oocytes. Furthermore, although the cleavage rate following ICSI with untreated and DTT-treated sperm was not significantly different, the rate of blastocyst formation was significantly higher in the DTT-treated sperm than in the untreated sperm.

2. 3. Physiological changes in bovine oocyte during maturation and fertilization

During resumption of meiosis, oocytes undergo a series of nuclear and cytosolic

changes that prepare them for fertilization and are referred to as oocyte maturation. These events include germinal vesicle breakdown (GVB), chromatin condensation and spindle formation and, among cytosolic changes, organelle redistribution and maturation of Ca^{2+} release mechanisms. Coordinated progression and completion of these changes during maturation ensure proper fertilization and initiation of development (Chang *et al.*, 1997).

Ca^{2+} release has been shown to participate in meiotic and mitotic transitions in numerous cell types (Whitaker and Patel, 1990). In mammalian oocytes and eggs, intracellular Ca^{2+} oscillations are associated with fertilization and activation of development. However, the role of intracellular Ca^{2+} oscillations during mammalian oocytes maturation is much less well understood (Homa, 1995).

2. 3. 1. Fluctuations in intracellular Ca^{2+}

It is generally believed that sperm-egg fusion somehow causes a large release of Ca^{2+} from intracellular sites, e.g., endoplasmic reticulum (Jaffe, 1983). This intracellular Ca^{2+} release triggers a Na^{+}/H^{+} exchange through the egg plasma membrane, resulting in a transient increase in intracellular pH. This temporal pH increase seems to remove inhibitory proteins in the egg cytoplasm, resulting in irreversible activation of the egg's oxidative pathways, lipid metabolism, and protein and DNA syntheses. In electrically non-excitable cells, Ca^{2+} oscillations, at least in mouse oocytes, appear to be mediated by the inositol 1,4,5-trisphosphate receptor ($InsP_3R$). The $InsP_3R$ is thought to mediate spontaneous oscillations during maturation in mouse oocytes (Fissore *et al.* 1998).

A transient calcium increase at fertilization is involved in the induction of cortical

granule (CG) exocytosis, in which thousands of cortical granules just beneath the egg plasma membrane fuse with the plasma membrane, allowing the contents of the cortical granules to be released into the perivitelline space. The contents of the cortical granules, which include various enzymes, enter the zona pellucida, causing it to harden. This reaction, called the zona reaction, occurs several minutes after fertilization. The reaction causes the zona pellucida to lose its ability to bind sperm, resulting a final block to polyspermy.

The pattern of Ca^{2+} release in mammalian oocytes is unique in that occurs in the form of oscillations that may last for up to 20 h (Miyazaki *et al.*, 1993; Nakada *et al.*, 1995). Completion of meiosis is caused by a series of repetitive Ca^{2+} transient increases triggered by the sperm at fertilization. Ca^{2+} oscillations in response to sperm occur after germinal vesicle breakdown but not during interphase. These transient increases in Ca^{2+} cease during entry into interphase, at the time when pronuclei are forming (Jones *et al.*, 1995b). During the steady-state period of sperm-induced Ca^{2+} oscillations, each individual intracellular Ca^{2+} spike invariably begins from a focus on the oocyte periphery and spreads throughout the entire peripheral region before propagating to the central ooplasm. This peripheral Ca^{2+} wave is immediately followed by a large intracellular Ca^{2+} increase in the ooplasm. However, this central Ca^{2+} rise only peaks when Ca^{2+} in the peripheral ooplasm is already on the decline. Moreover, the peak Ca^{2+} value is always considerably higher in the oocyte center than in the periphery (Tesarik *et al.*, 1995).

A large release of intracellular Ca^{2+} occurs in the hamster egg 10 to 30 sec after attachment of a fertilizing spermatozoon to the egg plasma membrane. Ca^{2+} release begins near the sperm attachment site. It spreads throughout the egg within 4 to 7 sec and

ends 15 to 20 sec later (Miyazaki *et al.*, 1986). Interestingly, the Ca^{2+} explosion occurs repeatedly, at regular intervals of 2 min and lasts for a period of 60 min (Miyazaki, 1991). In mature mouse eggs at fertilization, an initial large Ca^{2+} rise with a mean duration of 2.3 min is followed by several smaller rises at 3.4 min intervals (Fissore *et al.*, 1992), and the periodic increase in Ca^{2+} persists for 1 to 3 h (Shiina *et al.*, 1993; Jones *et al.*, 1995a). Immature oocytes generate only two or three oscillations, which cease within 1 h (Jones *et al.*, 1995a). Cytosolic Ca^{2+} increases, as seen in fertilized mouse and hamster eggs can be generated and perpetuated either by Ca^{2+} release from internal stores or by opening Ca^{2+} channels in the plasma membrane (Tsien and Tsien, 1990). In human oocytes at fertilization, Ca^{2+} oscillations last for up to 120 sec, and occur every 10 to 35 min (Taylor *et al.*, 1993). Ca^{2+} oscillations have also been observed in human oocytes fertilized by intracytoplasmic sperm injection (ICSI), but only after a considerable delay, which ranged between 25 and 88 min (Tesarik and Testart, 1994). While Ca^{2+} release is minimal in bovine oocytes for the first 15 h of maturation, a significant increase is observed between 15 h and 22 h after collection (Chang *et al.*, 1997). In unfertilized bovine eggs, the starting baseline levels of intracellular Ca^{2+} concentration is approximately 100*n*M and remains consistently at baseline values for 5 h (Fissore *et al.*, 1992).

After fertilization, three different intracellular Ca^{2+} patterns are observed during fertilization in bovine eggs. The most frequently detected sperm-induced Ca^{2+} change is a single elevation. The mean baseline Ca^{2+} concentration in eggs with Ca^{2+} elevations is 56 ± 11*n*M (mean ± SD). Peak Ca^{2+} concentration in eggs with a single elevation is 870 ± 184*n*M, which is higher than the 420 ± 27*n*M mean peak levels observed for eggs with

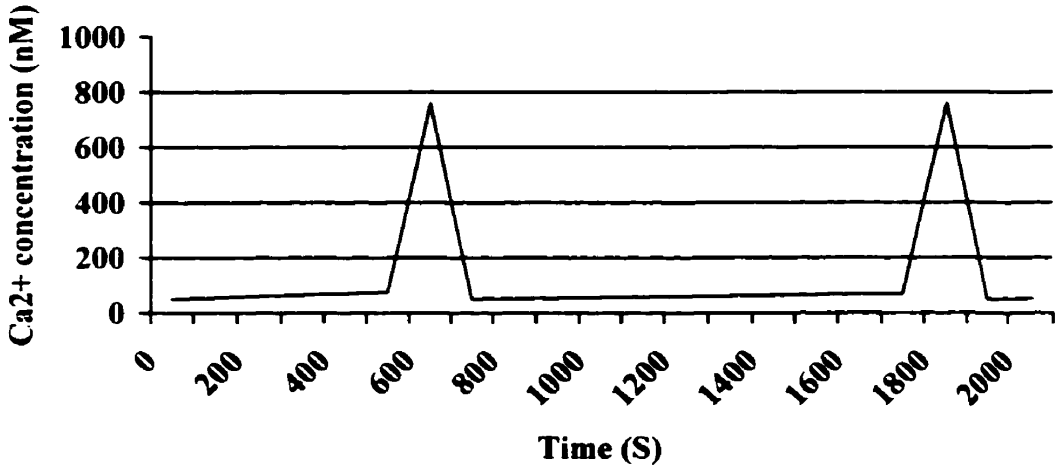
short-interval elevations. The mean duration of the Ca^{2+} elevations is 166 ± 13 sec and is different between groups (Fissore *et al.*, 1992). Sun *et al.* (1994) reported that after sperm penetration, a characteristic pattern of transient intracellular Ca^{2+} increase both with respect to spike amplitude and spiking interval was exhibited. In addition, several hours elapse before the first transient increase since the time required for sperm capacitation is longer in cattle than in other species. During the first 9 h after insemination (about 3~5 h after sperm penetration), the number of calcium spikes per oocyte observed is 2.8 ± 1.6, and the interval between spikes is 24.3 ± 7.3 min (Figure 1).

In mammalian oocytes, fertilization-associated intracellular calcium oscillations are responsible for the activation of development. The mechanism(s) by which the sperm triggers the initial intracellular Ca^{2+} rise and supports long-lasting oscillations is not resolved. It has been proposed that the sperm may interact with receptors in the oocyte's plasma membrane and engage intracellular signaling pathways that result in Ca^{2+} release.

2. 3. 2. Changes in histone H1 kinase

Mature mammalian oocytes are arrested in metaphase II of meiosis by a maturation-promoting factor (MPF). MPF consists of two sets of proteins, cyclins and $p34^{cdc2}$ (Labbe *et al.*, 1989), and $p34^{cdc2}$ is essential for the transitions from G1 to S and G2 to M phase in the cell cycle. Activation of the $p34^{cdc2}$ kinase requires cyclin, and the kinase activity of $p34^{cdc2}$-cyclin complexes is regulated by the phosphorylation state of both components (Murray *et al.*, 1989). Late in interphase, $p34^{cdc2}$ becomes complexed with cyclin, and, at the G2/M transition, dephosphorylation of the $p34^{cdc2}$/cyclin complex

Figure 1. The pattern of intracellular Ca2+ concentration in fertilized bovine eggs (Sun et al., 1994)

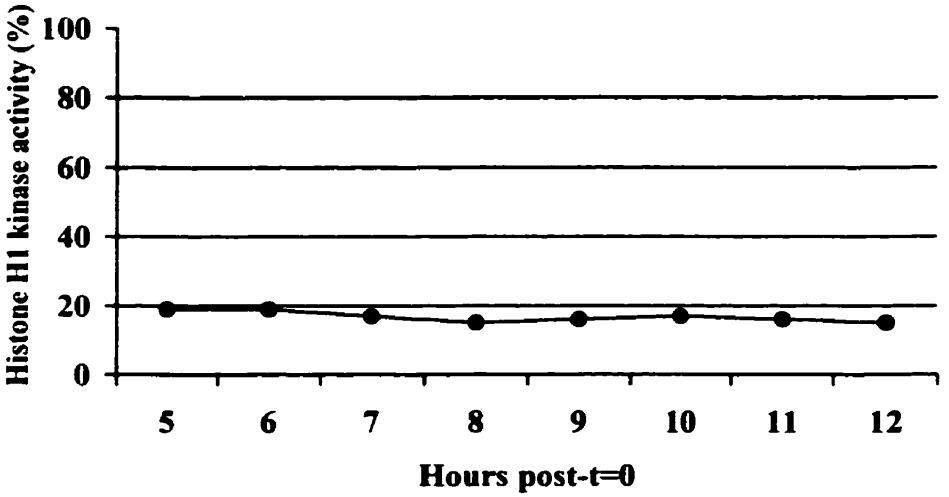


provides $p34^{cdc2}$ with histone H1 kinase activity. The kinase activity of MPF elicits a cascade of Ca^{2+}-dependent reactions leading to germinal vesicle breakdown, chromosome condensation, spindle formation, and entry into metaphase II (MII) arrest in the immature mammalian oocyte (reviewed in Collas *et al.*, 1995).

Fertilization or artificial activation of oocytes results in release from meiotic arrest by inactivation of MPF. Release from metaphase II is associated with loss of histone kinase activity. Fertilization triggers periodic Ca^{2+} elevations in oocytes, and these transient Ca^{2+} increases seem to be required for MPF inactivation, which is the result of abrupt cyclin degradation and $p34^{cdc2}$ phosphorylation (Whitaker and Patel, 1990). Inactivation of histone H1 kinase in oocytes allows resumption of meiosis and entry into the first embryonic cell cycle. Histone H1 kinase inactivation is dependent on extracellular Ca^{2+}, and histone H1 kinase is regulated by the number of Ca^{2+} stimulations. Ca^{2+} rises and histone H1 kinase interact at a critical point during fertilization; the Ca^{2+} response induced by the sperm during fertilization induces the decline in kinase activity that is required to exit meiosis (Chang *et al.*, 1997). Histone H1 kinase activity in fertilized oocytes decreases to 19 ± 1.2% of its original level at 5 h after insemination, and no fluctuations in activity occur for at least 8 h (Collas *et al.*, 1993b, Figure 2).

The decrease in histone H1 kinase activity can be triggered by a single Ca^{2+} stimulation. Histone H1 kinase is inactivated to 44 ± 11.5% of its initial level in 1 h, however histone H1 kinase inactivation is transient, and activity starts to increase at 2 h after a single Ca^{2+} stimulation. At 4 h, rate of increase is accelerated and the level of activity at 6 h is 112 ± 22.5% of that in MII oocytes (Campbell *et al.*, 1993; Collas *et al.*, 1993b). Therefore, although a single Ca^{2+} stimulation is sufficient to trigger histone H1

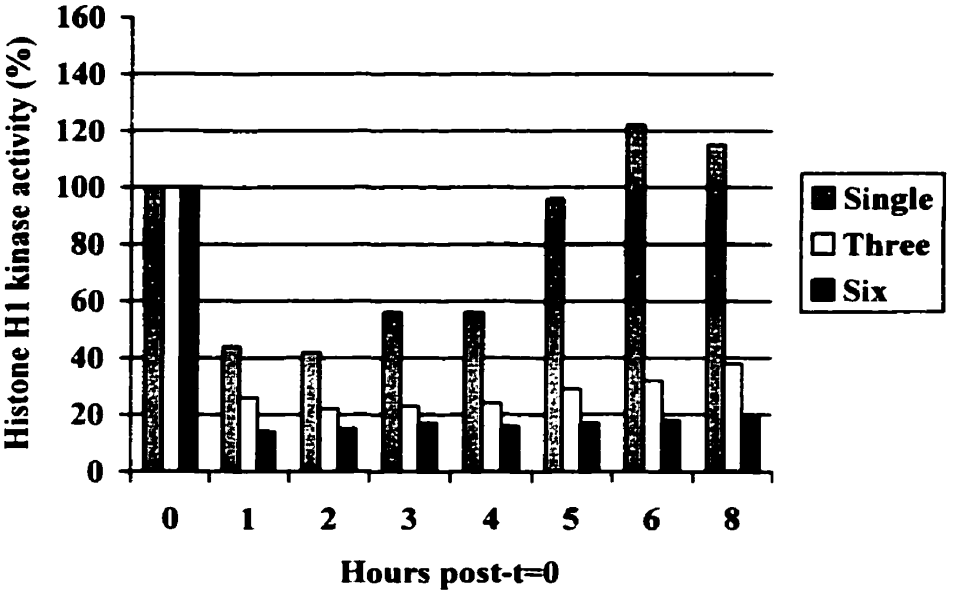
Figure 2. Basal histone H1 kinase activity in in vitro-fertilized oocytes (Collas et al., 1993b)



kinase inactivation, histone H1 kinase becomes reactivated after the initial inactivation. Reactivation of histone H1 kinase prematurely in the cell cycle may have detrimental effects on embryo development. The property of active MPF is to induce chromosome condensation. Histone H1 kinase reactivation early in the cell cycle, therefore, may induce premature condensation of the embryonic chromatin (PCC). After a single Ca^{2+} stimulation, PCC may induce the formation of metaphase III, when the activation stimulus was not sufficient to induce pronuclear formation. In addition to PCC, other events associated with chromatin condensation may also occur under increasing H1 kinase activity, which may also be responsible for impaired development. Reactivation of H1 kinase early in the first cell cycle, therefore, may have adverse effects on embryo development.

The onset of kinase reactivation is delayed, and the extent of reactivation is reduced, as the number of Ca^{2+} stimulations increase (Collas *et al.*, 1993b, Figure 3). A single stimulation is sufficient to initiate H1 kinase inactivation; however, subsequent Ca^{2+} stimulations are required to maintain basal H1 kinase activity. One of the functions of the multiple transient Ca^{2+} increases at fertilization is to maintain MPF in its inactive form, and transient Ca^{2+} increases are responsible for cell cycle onset, stimulation of DNA synthesis, and $p34^{cdc2}$ phosphorylation (Patel *et al.*, 1989). Therefore, it is likely that multiple Ca^{2+} elevations during artificial activation by electrical stimulation, elicited by multiple Ca^{2+} stimulations, are responsible for keeping MPF and histone H1 kinase inactive. When three Ca^{2+} stimulations are given, each 22 min apart, histone H1 kinase inactivation occurs within 1 h of the first Ca^{2+} stimulation, which corresponds to loss of approximately 75 ± 3% of initial kinase activity, and histone H1 kinase activity decreases

Figure 3. Histone H1 kinase activity from 0 to 8 h after a single, three and six Ca2+ stimulations (Collas et al., 1993b)



to basal levels within 30 min of the stimulation. Following this rapid decline, histone H1 kinase activity remains at ~20 ± 2.5% of its original level for 3~4 h. However, at 4~5 h after the first stimulation, histone H1 kinase activity starts to rise gradually until at least 8 h, when it reaches a mean value of 38 ± 15% of initial activity. Therefore, histone H1 kinase is rapidly inactivated after the initial stimulation and becomes reactivated 5 h after the first stimulation because active MPF is responsible for increasing histone H1 phosphorylation 5 h after initial stimulation (Collas *et al.*, 1993b).

Six Ca^{2+} stimulations are able to maintain basal histone H1 kinase activity for at least 8 h after the first stimulation. Histone H1 kinase activity drops to 14 ± 0.4% of its initial level within 1 h of the first stimulation and remains at this basal level (14~19%) until at least 8 h. Thus, after the six stimulations, no histone H1 kinase reactivation occurs for the first 8 h after stimulation. Therefore, the number of Ca^{2+} stimulations can regulate onset and amplitude of histone H1 kinase reactivation in stimulated oocytes (Collas *et al.*, 1993b). However, parthenogenetic development is low with this treatment (Collas *et al.*, 1993a). Although basal histone H1 kinase activity is similar to that of fertilized oocytes, it is likely that a detrimental effect, related or not to MPF, is caused by this activation procedure. Reactivation of MPF at this stage of the cell cycle would be harmful for development. Alternatively, these oocytes may have been overstimulated. Although Ca^{2+} overstimulation may not affect basal MPF activity, excessive intracellular Ca^{2+} has been shown to affect cellular functions and, thus, may affect the oocyte activation pathway (Collas *et al.*, 1993b).

2. 4. Activation of bovine oocytes

Oocyte activation is defined as a resumption of the second meiotic division; i.e., oocytes that have progressed to anaphase II, telophase II, metaphase III, or pronuclear formation are considered activated. Oocyte activation is normally triggered by the sperm. Without this stimulus, the oocyte would be unable to form pronuclei and become a zygote. At activation, the ooplasm shrinks in volume, expelling fluid into the perivitelline space. At the same time, the head of the spermatozoon in the ooplasm swells and acquires the consistency of a gel, losing its characteristic shape. The finai structure, which resembles the nucleus, is termed the male pronucleus. Artificial activation of a bovine oocyte results in term development following electrical stimulation in *in vitro* nuclear transplantation (Prather *et al.*, 1987) or Ionomycin A23187 activation in *in vitro* fertilization following microinjection of spermatozoa (Goto *et al.*, 1990). However, since the ability of the manipulated embryos to develop to term is very poor, a number of investigators using a parthenogenetically activated oocyte model have tried to improve the developmental ability of oocyte activated by stimulation other than spermatozoa.

2. 4. 1. Artificial stimulators for oocyte activation

Sperm-oocyte fusion induces a series of increases in the intracellular free Ca^{2+} concentration and the decline in histone H1 kinase activity in mammals. Most of the events associated with oocyte activation are the result of the increased Ca^{2+}, cortical granule (CG) exocytosis, resumption of meiosis, and cell division. All of these events are important for normal fertilization and early development. Many artificial stimulators, such as ethanol, Ionomycin (A23187), and an electrical pulse (First *et al.*, 1992; Sun *et*

al., 1992; Procházka *et al.*, 1993), can mimic fertilization to induce intracellular Ca^{2+} concentration increase and thus result in CG exocytosis, pronuclear formation, and early development.

To date bovine oocytes have been successfully activated by ethanol (Nagai, 1987), an electrical pulse (Kono, *et al.*, 1989), Ionomycin (Ware *et al.*, 1989), or cycloheximide (CHX) treatment (Sirard *et al.*, 1989). Among these options, an electric pulse seems to be the most frequently used artificial stimulus for bovine oocytes, in nuclear transfer experiments (Prather *et al.*, 1987). However, its efficacy depends upon, among other factors, the quality of the electroporation medium, the optimal combination of field strength and pulse duration, the correct placement of oocytes between wire electrodes, and the size of chamber used. In addition, electric stimulation inflicts damage to the spindle apparatus, resulting in its splitting. This causes aberrant chromatin migration and formation of multiple pronuclei. Chemical stimuli such as ethanol, CHX, or Ionomycin, on the other hand, are not limited by the more or less technical difficulties peculiar to electric stimulation (Soloy *et al.*, 1997a). Among these chemicals, Ionomycin is one of the most effective artificial stimulators that has been widely used in the activation of oocytes in animals. Because most of the events related to oocyte activation are calcium-dependent, and it is generally thought that Ionomycin directly induces the influx of extracellular calcium. Ionomycin may represent a very useful tool for investigating calcium-dependent processes in oocyte activation. However, the effectiveness of different stimulators is dependent upon the animals, the composition of medium used, and the age of oocytes. In pig oocytes, the electrically activated oocytes show the same penetrability by spermatozoa after *in vitro* insemination as that in control

oocytes (Wang *et al.*, 1998b), but Ionomycin-treated oocytes show significantly lower penetrability in a concentration dependent manner. It is possible that the CG exocytosis induced by electrical pulses does not activate CG enzymes, or destroys the bioactivity of CG materials, because Ionomycin and an electrical pulse induce the same degree of CG exocytosis. However, in bovine oocytes, electrically pulsed oocytes resist sperm penetration (Soloy *et al.*, 1997b). According to studies in bovine oocytes, histone H1 kinase activity is reduced at 30~60 min after an electrical pulse (Collas *et al.*, 1993b) and by 5 h after Ionomycin treatment (Soloy *et al.*, 1997a). Transient calcium increases are also different for the two stimulators in pig oocytes. Electrical pulses induced very fast and high Ca^{2+} transient increase, but Ionomycin A23187 induced a small Ca^{2+} transient increase. In addition, most of electrically pulsed oocytes do not release a second polar body and are penetrable by sperm, whereas Ionomycin-treated oocytes release a second polar body and are not penetrable by sperm. Sperm-penetrated oocytes also release a second polar body and are not penetrable by sperm after reinsemination. Therefore, the mechanisms are completely different for electrical activation and Ionomycin activation, with Ionomycin more closely mimicking sperm penetration (Wang *et al.*, 1998a).

Studies in cattle with ethanol (Nagai, 1987, 1993; Yang *et al.*, 1994) or Ionomycin (Ware *et al.*, 1989; Soloy *et al.*, 1997a) indicate that the activation rates are dependent upon the age of oocytes. Nagai (1987) reported that bovine oocyte activation with ethanol approaches peak efficiency around 27 h of maturation *in vitro*. The time course for responsiveness to ethanol activation is similar to the time course reported by Ware *et al* (1989) for both Ionomycin and electrical pulse. However, although activation rates are higher in aged oocytes than in young oocytes, it is generally accepted that aged

oocytes are beyond their normal fertilizable life span at the moment of artificial stimulation. Therefore, it is reasonable to expect that such activated oocytes will be less viable. According to the study by Hagen *et al* (1991), oocyte age-dependent activation of pig oocytes is not observed by electric pulse. It is possible that the mechanisms of oocyte activation by Ionomycin or other artificial stimulations are different among species.

Recently, a number of investigators have examined the synergistic effect of combination of Ionomycin with an inhibitor of protein phosphorylation, 6-dimethylaminopurine (DMAP) (Susko-Parrish *et al.*, 1994) or of protein synthesis inhibitor, cycloheximide (CHX) (Soloy *et al.*, 1997a) on parthenogenic activation of bovine oocytes. Oocytes treated with Ionomycin alone activate a high proportion of the oocytes (57.8%) by 1 h, and there is evidence of second polar body expulsion in oocytes as early as 2 h after Ionomycin. However, after completing meiosis instead of forming pronuclei (8.9%), the majority of chromosomes remain condensed in metaphase III (metaphase III is an abnormal stage in which chromosomes remain condensed after telophase II due to insufficient ooplasmic activation). In contrast, when oocytes are exposed sequentially to Ionomycin and DMAP, there is evidence of pronuclear formation (80.5%) by 2 h and maximum response by 3 h. No signs of meiotic resumption or second polar body expulsion are observed in oocytes with sequential treatment (Susko-Parrish *et al.*, 1994). The DMAP treatment causes the second meiotic spindle to disintegrate, the oocytes go directly into interphase, and only one diploid pronucleus is formed. DMAP has been shown to inhibit phosphorylation but not inhibit protein synthesis. It appears to work by inhibiting protein kinase and not by stimulating phosphatases. The role of DMAP may indirectly inhibit the activity of maturation promoting factor (MPF) allowing

the oocytes to escape meiosis and re-enter interphase. DMAP may also be inhibiting phosphorylations necessary for the spindle apparatus thus inhibiting both meiotic resumption and second polar body expulsion of second reduction division. Navara *et al.* (1994) examined parthenogenic activation rates with activated mature metaphase II-arrested oocytes (24 h in culture) using one of two methods; two 5-min incubations in 5μM Ionomycin in culture medium given 4 h apart or one 5-min incubation in 5μM Ionomycin followed by a 4 h incubation in 1.9mM 6-dimethylaminopurine in culture medium. These methods successfully activate >80% of mature bovine oocytes. Oocytes activated by the first method normally extrude a second polar body and are, therefore, haploid. Four percent of these parthenogenotes develop to the blastocyst stage, arresting along each stage of development. The second treatment using Ionomycin followed by DMAP induces the formation of a single diploid pronucleus. Forty percent of oocytes activated in this manner develop to the blastocyst stage. It is likely that the improved development rate is due to result diploidy rather than method of activation.

3. GENERAL OBJECTIVE

The major objective of this study was to identify and investigate factors limiting successful intracytoplasmic sperm injection (ICSI) with the aim of developing a reliable method for ICSI in cattle. As a consequence, a series of experiments were designed which focused on oocyte activation.

To achieve this goal the following secondary objectives were set to:

1) evaluate bull semen used in all ICSI experiments using a standard IVF procedure
2) determine the effect of centrifugation of bovine oocytes on the efficiency of ICSI
3) examine embryonic development to blastocyst stage in bovine oocytes treated with Ionomycin or Ionomycin and DMAP following ICSI
4) determine if the activation of oocytes with Ionomycin and DMAP following ICSI induces normal pronuclear formation
5) determine the effect of multiple stimulations and different concentrations of Ionomycin on activation of bovine oocytes following ICSI

4. MATERIALS AND METHODS

4. 1. *In vitro* maturation, fertilization and development

4. 1. 1. Collection and *in vitro* maturation of oocytes

Bovine ovaries were obtained from an abattoir and transported to the laboratory in 0.9% physiological saline at 25°C to 28°C within 3 h of slaughter. Follicular oocytes were recovered by aspirating follicles of 2 to 6 mm diameter with an 18-gauge needle. Only oocytes surrounded by compact and dense cumulus cell layers were selected. These were washed through three rinses of HEPES-buffered TCM 199. Cumulus-oocyte complexes were placed in 25mM HEPES-buffered TCM 199 (Earle's Salt; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Gibco), 1μl/ml gentamycin (Gibco), 22μg/ml sodium-pyruvate (Sigma, St. Louis, MO., U.S.A.), 0.5μg/ml FSH (Folltropin V®, Vetrepharm, Inc., London, Canada), 50μg/ml hCG (APL®, Ayerst Laboratories, Montreal, Canada) and 1μg/ml 17β-estradiol (Sigma). Oocytes were matured in groups of 10, in 50μl droplets covered with mineral oil (Sigma) at 39°C in an atmosphere of 5% CO_2 in humid air for 22 ~24 h.

4. 1. 2. *In vitro* fertilization

The sperm that were used for this experiment were from the frozen semen of a single bull (Laurent, 073H01197) that was acquired from the Centre d'insémination

artificielle du Québec (C.I.A.Q) Inc. Sperm were separated by the method of Parrish *et al.* (1995). One 0.25ml straw of frozen semen was thawed in water at 37°C for 10 sec. The contents of the straw were placed on a discontinuous gradient of 45% and 90% Percoll (Sigma). The gradient consisted of frozen-thawed semen layered over 2 ml of 45% Percoll and 2 ml of 90% Percoll in a 15 ml conical plastic tube. The gradient and sperm were centrifuged at 857 × g for 30 min. The sperm pellet from the 90% Percoll fraction was then resuspended in fertilization medium (Lim *et al.*, 1994; BECM: Bovine Embryo Culture Medium, Table 1) supplemented with 6mg/ml bovine serum albumin (BSA, fatty acid free, Sigma) and 1µl/ml gentamycin.

Oocytes with expanded cumulus after in vitro maturation were used for in vitro fertilization. To induce capacitation of sperm, 0.25mg/ml heparin (Sigma, 25,000 units) and 2.5µg/ml hypotaurine (Sigma) were added in fertilization medium. Sperm were counted in a hemocytometer and checked for motility (i.e., at least 80% progressively motile); then oocytes were inseminated with a final concentration of 1×10^6 cells/ml.

Sperm and cumulus-oocyte complexes were incubated for 18 h at 39°C in 5% carbon dioxide in air. After washing three times in HEPES-buffered TCM 199, the presumptive zygotes were transferred to 50µl droplets of development medium (BECM + 3mg/ml BSA + 1µl/ml gentamycin) under mineral oil. Medium in each droplet was changed at 48 h and again 96 h; at 120 h, zygotes were placed in droplets of BECM supplemented with 10% FCS and 22µg/ml sodium-pyruvate.

Proportions of inseminated oocytes reaching blastocysts were recorded at 168 h. Expanded blastocysts and hatching were recorded at 216 h postinsemination.

Table 1. Composition of BECM (Lim *et al.*, 1994)

Compounds	mM
NaCl	89.00
KCl	3.20
$CaCl_2 \cdot 2H_2O$	2.00
$MgCl_2 \cdot 6H_2O$	0.50
$NaHCO_3$	25.00
NaH_2PO_4	0.35
Sodium lactate	10.00
Sodium pyruvate	0.50
BME amino acids (100x)	10.00 ml/L
MEM amino acids (100x)	10.00 ml/L
L-glutamine	1.00
Phenol red	0.001 g/L

pH : 7.2~7.3
260 mOsm.

4. 1. 3. Parthenogenetic activation

Cumulus cells were normally removed from oocytes at 22~24 h by vortexing for 2 min in 1mg/ml hyaluronidase (Type 1-S: From Bovine Testes, Sigma) dissolved in HEPES-buffered TCM 199. Denuded oocytes were activated as described by Susko-Parrish *et al.* (1994) with or without centrifugation of oocytes at 6,000 × g for 5 min. Those oocytes were exposed to 5μM Ionomycin A23187 (5mM stock in DMSO, Sigma) for 5 min in HEPES-buffered TCM 199 supplemented with 1mg/ml fatty acid free BSA and 112μg/ml sodium pyruvate (Handling Medium: HM). Oocytes were then moved to HM with 30mg/ml fatty acid free BSA to stop activation followed by a 4-h incubation in 1.9mM 6-dimethylaminopurine (DMAP, Sigma) in development medium. The cleavage rate and blastocysts were determined at 24 h and 168 h after activation, respectively.

4. 2. Intracytoplasmic sperm injection (ICSI)

4. 2. 1. Oocyte preparation

Following maturation for 22~24 h, cumulus-oocyte complexes were stripped of their cumulus cells by vortexing for 2 min in 1mg/ml hyaluronidase dissolved in HEPES-buffered TCM 199 and then washed in HEPES-buffered TCM 199. Denuded oocytes with a spherical shape, a visible first polar body (Pb1) and an evenly granulated ooplasm were subsequently centrifuged at 6,000 × g for 5 min to facilitate sperm injection (Figure 4) (Rho *et al.*, 1998). The centrifuged oocytes were transferred to droplets of HEPES-

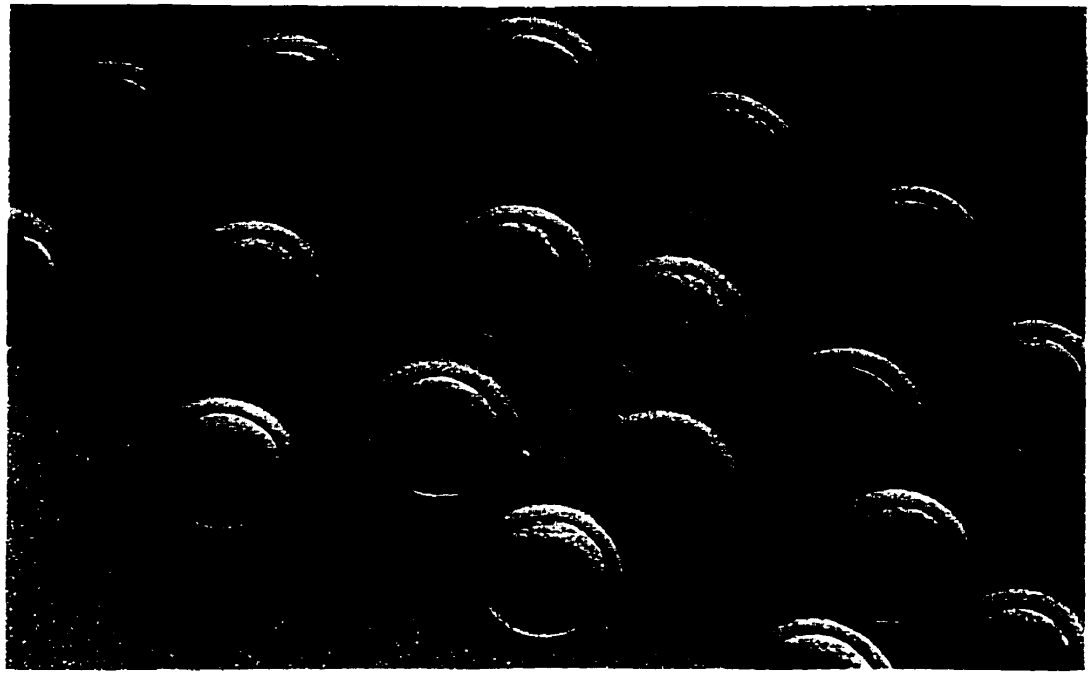


Figure 4. The oocytes centrifuged at 6,000×g for 5 min to facilitate sperm injection

buffered TCM 199 supplemented with 1mg/ml BSA before sperm injection.

4. 2. 2. Sperm preparation

After sperm treatment with a discontinuous gradient of 45% and 90% Percoll, a 0.1ml aliquot was layered under 0.8ml fertilization medium in a small culture tube supplemented with 0.1ml of 5mM dithiothreitol (DTT, Sigma), which is a disulfide reducing agent (Perreault and Zirkin, 1982; Rho *et al.*, 1998). One hour after incubation, the sperm that had swum up into the top 0.5ml in the culture tube were washed twice by suspension and centrifugation at 350 × g for 10 min in 10ml fertilization medium to remove DTT. The sperm pellet was resuspended in 1ml fertilization medium and these sperm were used for injection.

4. 2. 3. Preparation of oocyte-holding and injection pipettes

The oocyte-holding pipette (Borosilicate glass capillary tubing; 1.0mm o.d., 0.75mm i.d.; Sutter Instruments, Novato, CA) was drawn by hand, and the pipette end was polished with a microforge (Model MF-1; Technical Products International Inc., USA). The inner and outer diameter of the holding pipette were approximately 40μm and 180μm, respectively. Each injection pipette was drawn with a pipette puller (Model P-87; Sutter Instruments), and the tip of the pipette was ground at a 40° angle with a micro-grinder (Model EG-4; Narishige, Tokyo, Japan). The tip of the pipette was made sharply pointed with a microforge. The inner diameter near the pipette tip was approximately

8~9μm. To thin the glass wall of the pipette, the pipette tip was immersed in a 25% solution of hydrofluoric acid (Sigma) while the solution was drawn in and blown out of the pipette several times. The pipette was thoroughly rinsed with distilled water, and dried. The pipette was filled with fluorinert (Sigma), and then connected to a water-filled micrometer syringe system in a Narishige micromanipulator.

4. 2. 4. Sperm injection

Sperm injection was performed using a Carl Zeiss Sedival inverted microscope with Narishige micromanipulator (NT-88; Narishige, Tokyo, Japan). Sperm suspensions were diluted approximately 1:5 with 5% polyvinylpyrrolidone (PVP, Mol. Wt. 360,000, Sigma) in 0.9% saline solution (Perreault and Zirkin, 1982). A row consisting of two round droplets and one elongated droplet was placed along the centerline of the dish (Falcon #1001 100 × 15mm). The first droplet (10μl) was for pipette washing (PVP-saline). The second droplet (10μl) was the sperm suspension in PVP-saline. The third elongated droplet (150μl) was HEPES-buffered TCM 199 supplemented with 1mg/ml BSA (Handling medium) for the oocytes. These droplets were covered with mineral oil.

Approximately six partially denuded oocytes were placed in the droplet of handling medium. An individual sperm cell was picked up from the droplet of sperm and PVP mixture by aspirating the tail first at the bottom of dish (Figure 5), and then moved to the droplet containing the oocytes to be injected. An oocyte was held to the holding pipette at the 9 o'clock position, with the first polar body being around either the 12 or 6 o'clock position. There are two reasons for the positioning used. First, such positioning

would avoid damage to the metaphase II spindle that could be caused by deep insertion of a pipette into the oocyte. Second, the oocyte cortex in the vicinity of the metaphase spindle is very tough, probably because of the presence of abundant actin filaments (Maro *et al.*, 1986). After the injection pipette containing a spermatozoon was inserted into the ooplasm at 3 o'clock (Figure 6, 7), a moderate vacuum was established in order to rupture the oolemma and suck some of the ooplasm into the injection pipette (Figure 8). Subsequently, the aspirated ooplasm and spermatozoon were expelled into the ooplasm with a minimum volume of medium. The sperm-suspending medium was retrieved to the extent possible, with great care being taken not to extract an excessive amount of ooplasm. The pipette was withdrawn gently (Figure 9). One hour after injection, oocytes having the spermatozoon in the perivitelline space were removed from the experiment.

4. 3. Oocyte activation following ICSI

4. 3. 1. Oocyte activation with Ionomycin (Ca^{2+} Ionophore)

Injected oocytes were activated as described by Ware *et al.* (1989). The oocytes were washed twice in HEPES-buffered TCM 199 supplemented with 1mg/ml BSA (Handling Medium: HM) and then exposed to 5μM Ionomycin in HM for 5 min at 39°C. An equal volume of HM containing 30mg/ml BSA is added for 5 min at 39°C to stop the activation process. The activated oocytes were washed twice in HM and once in development medium.

4. 3. 2. Oocyte activation with Ionomycin & 6-dimethylaminopurine (DMAP)

The oocytes activated with 5μM Ionomycin were cultured for 3 h in development medium to permit extrusion of the second polar body (Pb2), and then transferred to a droplet of 1.9mM 6-dimethylaminopurine for an additional 3 h (Susko-Parrish *et al.*, 1994; Rho *et al.*, 1998). After washing twice in HM and once in development medium, the oocytes were cultured for 48 h in development medium and scored for cleavage rate and then transferred to fresh droplets of development medium for additional incubation, or they were fixed for observation.

4. 3. 3. Oocyte activation with multiple Ionomycin treatments at two concentrations

Injected oocytes were exposed to 5μM or 50μM Ionomycin in HM for three 5-min incubations at 30-min intervals. Initially, injected oocytes were washed twice in HM and then transferred to a 50μl HM droplet containing Ionomycin and incubated for 5 min at 39°C. After each 5-min incubation, an equal volume of HM containing 30mg/ml BSA was added for a further 5 min at 39°C to stop the activation process. The oocytes were then washed twice in HM and once in development medium prior to the next 30-min incubation in development medium. Following the third activation treatments, the activated oocytes were cultured for 16 h in development medium.

4. 4. Examination of oocytes

The oocytes cultured for 16 h were fixed by one of two methods: (1) in acetic acid: ethanol (1:3) and stained with 1% orcein dissolved in 40% acetic acid, and (2) in 1.25% glutaraldehyde in phosphate-buffered saline solution (PBS) containing 25μl Triton X-100 and stained with Hoechst 33342 glycerol solution (0.01mg Hoechst in 1ml PBS + 9ml glycerol) to examine pronuclear formation.

4. 5. Statistical analyses

Percentage data were subjected to arcsin transformation and the transformed data were analyzed by ANOVA using the statistical analysis system (SAS) statistical software package (SAS, Cary NC, 1990). Comparison of means among treatments was performed using Duncan's multiple range test. A p value <0.05 was considered to be statistically significant.

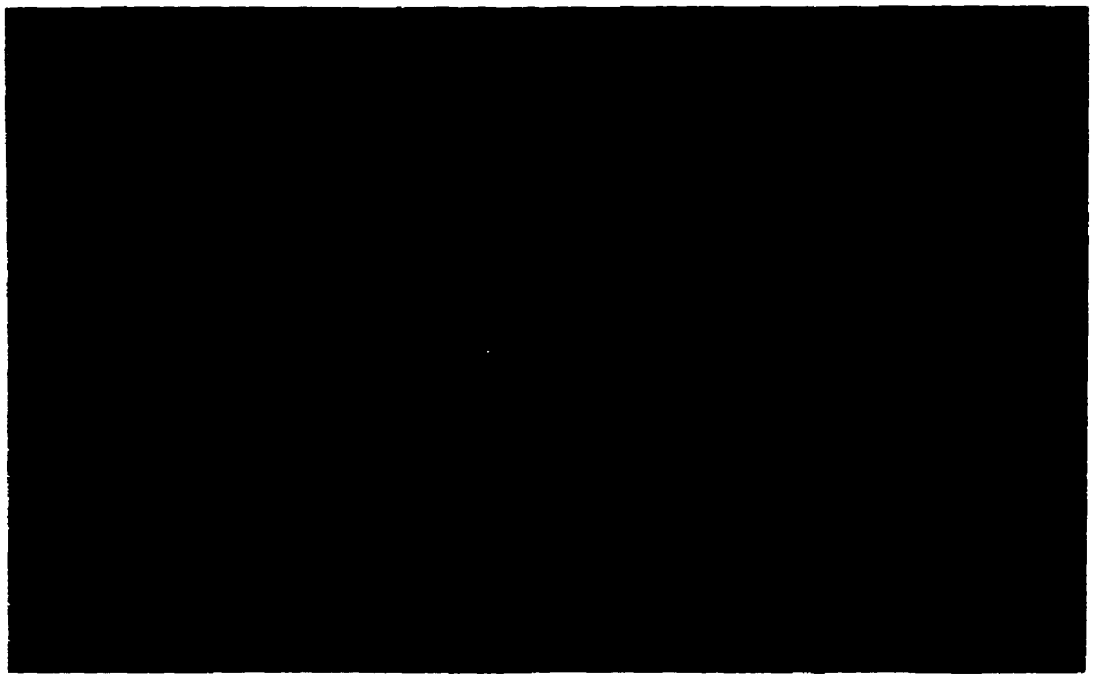


Figure 5. A spermatozoon was sucked, tail first, into injection pipette.

Figure 6. The injection pipette containing a spermatozoon was inserted into an oocyte.

Figure 7. The injection pipette was inserted deeply into the ooplasm.

Figure 8. A moderate vacuum was established in order to rupture the ooplemma and suck some of the ooplasm into the injection pipette.

Figure 9. The aspirated ooplasm and spermatozoon were expelled into the ooplasm, and the injection pipette was withdrawn gently.

5. EXPERIMENTAL PROCEDURES

5. 1. Experiment I : *In vitro* fertilization

5. 1. 1. Objective

To evaluate semen to be used in all ICSI experiments using a standard IVF procedure.

5. 1. 2. Experimental design

The semen that was used for this experiment was frozen semen from a single bull (Laurent, 073H01197) acquired from Centre d'insémination artificielle du Québec (C.I.A.Q) Inc. Cumulus-oocyte complexes were aspirated from abattoir-derived ovaries randomly. The cleavage rate was recorded at 24 h after *in vitro* insemination. Blastocysts were recorded at 168 h; expanded blastocysts and hatching, at 216 h postinsemination. After ten replicates were performed with the standard IVF technique, the mean of cleavage rate and blastocyst development was compared with the results provided by Boviteq Inc.

5. 1. 3. Result and discussion

The bull (Laurent 073H01197) whose semen was used for this experiment had a

56 day non-return rate of 76.9%. The cleavage rate and rate of blastocyst formation *in vitro*, as reported by Boviteq Inc., were 75.0% and 25~30%, respectively (Table 2).

Table 3 shows cleavage rate and blastocyst formation following *in vitro* fertilization (IVF). In our IVF system, 72.8% of inseminated oocytes cleaved and 22.3% formed blastocysts. Out of a total of 148 blastocysts, hatching occurred in 49 (33.1%), while early and expanded blastocysts were seen in 33 (22.3%) and 66 (44.6%), respectively. There was no significant difference in the cleavage and blastocyst rates obtained in our lab when compared with IVF results obtained by Boviteq Inc. using the same bull. These results demonstrated that our IVF system was acceptable and the semen that would be used in the ICSI experiments could direct normal fertilization.

5. 2. Experiment II : Effect of centrifugation on bovine oocytes

5. 2. 1. Objective

To determine the effect of centrifugation of bovine oocytes on the efficiency of intracytoplasmic sperm injection.

5. 2. 2. Experimental design

A total of 157 *in vitro* matured oocytes were assigned at random to one of two groups submitted or not to centrifugation at 6,000 × g for 5 min. Three replicates were carried out in each group. Oocytes were activated with 5μM Ionomycin for 5 min

Table 2. Blastocyst formation following *in vitro* fertilization (by Boviteq Inc.)

Bull	*56 day N.R. (%)	No. cleaved (%)	No. of blastocysts (%)
Laurent	76.9	75.0	25~30

*56 day Non Return Rate

Table 3. Blastocyst formation following *in vitro* fertilization

Number of replicates	Number of oocytes examined	Number of oocytes cleaved	Number of blastocysts			
			Early B	Expanded B	Hatching	Total
1	70	36	1	8	2	11
2	80	57	4	6	4	14
3	40	27	3	1	2	6
4	80	61	2	7	6	15
5	100	78	3	11	9	23
6	100	87	9	17	10	36
7	50	33	3	2	6	11
8	60	35	3	4	5	12
9	50	41	3	6	3	12
10	35	29	2	4	2	8
Total (%)	665	484 (72.8)	33	66	49	148 (22.3)

followed by a 4 h incubation in 1.9mM 6-dimethylaminopurine (DMAP). The cleavage rate and blastocyst development were determined at 24 h and 168 h as a measure of parthenogenic activation, respectively.

5. 2. 3. Result and discussion

Table 4 shows parthenogenic development of bovine oocytes following centrifugation. The cleavage rate of centrifuged and uncentrifuged control oocytes was 84.6% (66 of 78) and 91.4% (73 of 79), respectively. There was no significant difference between the two groups in cleavage rate. Also, centrifugation of oocytes had no detectable influence on development to blastocysts when compared with controls (18.0 vs. 20.3%) following parthenogenic activation. Oocytes from domestic species such as cow and sheep have more dense cytoplasm than those of mouse, hamster and human.

In our early experiments, this dark appearance of the cytoplasm of *in vitro* matured bovine oocytes made it difficult to determine whether or not the sperm had been successfully injected into oocytes and that the injection pipette had ruptured the ooplasmic membrane. To overcome these problems, *in vitro* matured bovine oocytes were centrifuged at 6,000×g for 5 min to displace the intracellular lipid inclusions to the periphery of the oocytes. High-speed centrifugation readily displaces intracellular lipid allowing the dark appearance of cytoplasm to be clarified. Therefore, centrifugation of bovine oocytes before ICSI makes it possible to see the spermatozoon as it is expelled from the injection pipette into the cytoplasm. Intracellular lipid inclusions displaced by centrifugation remained restricted to a small portion of the oocytes, and then intracellular

Table 4. Parthenogenic development of bovine oocytes following centrifugation.

Group	Treatment	[a]No. of oocytes	No. cleaved (%)	No. of blastocysts (%)
Uncentrifuged (control)	Ionomycin + DMAP	79	73 (91.4)[b]	16 (20.3)[b]
Centrifuged	Ionomycin + DMAP	78	66 (84.6)[b]	14 (18.0)[b]

[a] Done in three replicates
[b] Within columns, difference is not significant

lipid inclusions became redistributed throughout the ooplasm before the formation of pronuclei (within approximately 16 h). After centrifugation, oocytes were activated parthenogenetically with 5μM Ionomycin for 5 min followed by a 4 h incubation in 1.9mM 6-dimethylaminopurine (DMAP) to determine the effect of centrifugation on bovine oocytes. Our study demonstrated that the cleavage rate and development to blastocysts were not affected by centrifugation when compared with noncentrifuged control oocytes (84.6 vs. 91.4% and 18.0 vs. 20.3%, respectively), although comparison of cell numbers in blastocysts was not examined. This result agreed with the studies of Wall and Hawk (1988) which showed that centrifugation did not significantly influence development of cow zygotes. Therefore, this result suggests that centrifugation of *in vitro* matured bovine oocytes is a simple, reliable method that can be applied to the ICSI technique.

5. 3. Experiment III : Development of bovine oocytes after ICSI

5. 3. 1. Objective

To examine embryonic development to blastocyst stage in bovine oocytes treated with Ionomycin or Ionomycin and DMAP following injection with or without sperm.

5. 3. 2. Experimental design

A total of 316 oocytes were assigned to one of four group, which are treated with

Ionomycin or Ionomycin and DMAP following injection with or without sperm (sham injection). The experiment was conducted in six replicates. Injected oocytes were activated as described by Rho *et al.* (1998). The oocytes activated with 5μM Ionomycin were cultured for 3 h in development medium, and then transferred to a droplet of 1.9mM 6-dimethylaminopurine (DMAP) for an additional 3 h. The cleavage rate was examined at 24 h after activation, and the oocytes were scored every 48 h. Blastocysts were examined at 168 h after activation.

5. 3. 3. Result and discussion

Table 5 shows the cleavage rate and development of bovine oocytes following ICSI and activation with Ionomycin or Ionomycin and DMAP. The cleavage rate of sperm-injected group treated with 5μM Ionomycin and 1.9mM DMAP was higher than with Ionomycin alone (62.1 vs. 27.3%, $p<0.05$). In addition, the sperm-injected group treated with Ionomycin stopped development at an early stage (2~8 cell), whereas the sperm-injected group treated with Ionomycin and DMAP showed better development to the sixteen-cell stage. Moreover out of a total of 66 injected oocytes, 1 (1.5%) and 2 (3.0%) developed to the morulae and blastocyst stages, respectively. In sham-injected control group treated with Ionomycin and DMAP, the cleavage rate was approximately six times higher than that of oocytes treated with Ionomycin alone (44.3 vs. 7.4%, $p<0.001$). Sham-injected control group stopped development between the four and sixteen-cell stages.

The present study confirmed that the injection procedure itself will not induce

Table 5. Development of bovine oocytes following ICSI and activation with Ionomycin or Ionomycin and 6-dimethylaminopurine

Group	Treatment	[a]Number of oocytes examined	Number of oocytes cleaved(%)	No. (%) of oocytes developed to: 2cell	4cell	8cell	16cell	Mor.	Bla.
Sperm injection	Ionomycin	66	18 (27.3)[c]	8 (12.1)	5 (7.6)	3 (4.5)	2 (3.0)		
	Ionomycin + DMAP	66	41 (62.1)[b]	5 (7.6)	13 (19.7)	13 (19.7)	7 (10.6)	1 (1.5)	2 (3.0)
Sham injection (control)	Ionomycin	68	5 (7.4)[d]	3 (4.4)	2 (2.9)				
	Ionomycin + DMAP	70	31 (44.3)[bc]	10 (14.3)	11 (15.7)	7 (10.0)	3 (4.3)		

[a] Done in six replicates

[b,c,d] Values with different superscripts within a column are significantly different, $p<0.05$,

[bc,d] $p<0.001$.

pronuclear formation, and only 1 of 36 oocytes had one pronucleus in the untreated sperm-injected group. Keefer *et al.* (1990b) found that bovine oocytes are less sensitive to the injection process than are hamster or rabbit oocytes in that only a low percentage (2%) of bovine oocytes activated following injection, indicating that the ICSI technique, by itself, does not contribute to activation of bovine oocytes. Unlike hamster and rabbit, therefore, an oocyte activation procedure is essential for successful ICSI in cattle. Since most of the events related to oocyte activation are calcium-dependent, and it is generally thought that the Ca^{2+} ionophore, Ionomycin, directly induces the influx of extracellular calcium, Ionomycin has been widely used in the activation of oocytes in mammals. Recently, a number of investigators have examined the synergistic effect of combining Ionomycin with an inhibitor of protein phosphorylation, DMAP (Susko-Parrish *et al.*, 1994) or a protein synthesis inhibitor, cycloheximide (CHX) (Soloy *et al.*, 1997a) on parthenogenic activation of bovine oocytes. Rho *et al.* (1998) used Ionomycin and DMAP in a slight modification of the method reported by Susko-Parrish *et al.* (1994) to activate bovine oocytes following ICSI. For oocyte activation following ICSI, we initially used the method of Rho *et al.* (1998); the regimen with a 3-h interval between Ionomycin activation and DMAP treatment allowed time for the extrusion of the second polar body. In experiment III, we found that cleavage rate in both sperm-injected and sham-injected control groups treated with Ionomycin and DMAP was much higher than with Ionomycin alone (62.1 vs. 27.3%, $p<0.05$ and 44.3 vs. 7.4%, $p<0.001$, respectively). This result suggests that treatment combining Ionomycin and DMAP can efficiently activate bovine oocytes following ICSI. There was a significant difference between the two groups activated with Ionomycin alone (27.3 vs. 7.4%, $p<0.05$); hence, the higher cleavage rate

in sperm-injected oocytes suggests that sperm contribute to the activation process. The mechanisms by which fertilizing spermatozoa induce oocyte activation are much less well understood. However, a number of investigators have evidence that the spermatozoon activates oocytes by releasing a soluble, oocyte-activating substance into the oocyte. Recently, Kimura *et al.*, (1998) demonstrated that mammalian spermatozoa introduce active sperm-borne oocyte-activating factor(s) (SOAF) into oocytes to activate them. In the mouse, SOAF is located within the sperm head and appears during transformation of the round spermatid into the spermatozoon. Although the action of SOAF is not highly species-specific, it still remains ill defined in cattle. There was no significant difference in cleavage rate between the two groups treated with Ionomycin and DMAP. This result raises the question of the efficiency of DMAP on oocyte activation following ICSI. DMAP is one of the most effective phosphorylation inhibitors used in oocyte activation parthenogenetically. The DMAP treatment causes the second meiotic spindle to disintegrate, the oocytes go directly into interphase, and only a diploid pronucleus is formed. According to the Susko-Parrish *et al.* (1994) studies, treatments combining Ionomycin and DMAP result in a decrease in oocytes with one pronucleus and one polar body (uniformly diploid parthenogenones) and an increase in a mixture of diploid and haploid parthenogenones, since DMAP was capable of causing transition to interphase of all chromatin configurations after anaphase II commenced and prior to formation of the second polar body (2Pb). Therefore, although Ionomycin and DMAP treatment in sperm-injected oocytes resulted in a high cleavage rate and 3% blastocysts, it can not be concluded that oocytes treated with Ionomycin and DMAP following ICSI are able to form normal zygotes and subsequently develop to the blastocyst stage.

5. 4. Experiment IV : Pronuclear formation in bovine oocytes after ICSI

5. 4. 1. Objective

To determine if the activation of oocytes with Ionomycin and DMAP following ICSI induces normal pronuclear formation.

5. 4. 2. Experimental design

A total of 132 oocytes were assigned to one of two groups, one of which was injected with sperm and the other sham-injected. The experiment was conducted in five replicates. The oocytes activated with 5μM Ionomycin were cultured for 3 h in development medium, and then transferred to a droplet of 1.9mM DMAP for an additional 3 h. The oocytes were cultured for 16 h, and then were fixed and stained with orcein or Hoechst 33342 to examine for pronuclear formation.

5. 4. 3. Result and discussion

Upon examination 16 h after ICSI, pronuclear formation was observed in 33 of 47 (70.2%) Ionomycin and DMAP-treated oocytes. Two pronuclei were present in 18 of 33 (54.6%), while one and three pronuclei were seen in 8 of 33 (24.2%) and 7 of 33 (21.2%), respectively (Table 6). In sham-injected Ionomycin and DMAP-treated control group, out of a total of 15 with pronuclear formation, 6 of 15 (40.0%) had one pronucleus while 9 of

Table 6. Parthenogenic development of bovine oocytes following activation with Ionomycin and 6-dimethylaminopurine

Group	[a]Number of oocytes injected	Number of oocytes examined	Pronuclei (%)			
			1PN	2PN	3PN	Total
Sperm injection	66	47	8 (17.0)[b]	18 (38.3)[b]	7 (14.9)[b]	33 (70.2)[b]
Sham injection (control)	66	38	6 (15.8)[b]	9 (23.7)[b]	0 (0)[c]	15 (39.5)[c]

[a] Done in five replicates

[b,c] Values with different superscripts within columns are significantly different, $p<0.05$.

15 (60.0%) had two pronuclei. Three pronuclei were not observed in sham-injected control group. Although there was no significant difference between the two groups in one and two pronuclear formation rate, total pronuclear formation rate was significantly different between the two groups (70.2% vs. 39.5%, $p<0.05$).

In experiment IV, we found that total pronuclear formation was significantly different between the sperm-injected and sham-injected control groups (70.2 vs. 39.5%, $p<0.05$). Ionomycin and DMAP treatment permitted a high rate of development of normal zygotes (2PN) (18 of 33, 54.6%) in the sperm-injected group, although three pronuclei (3PN; abnormal) were also observed in 7 of 33 (21.2%) in this group. Furthermore, out of a total of 15 sham-injected control oocytes with pronuclear formation, 9 of 15 (60%) had two pronuclei (2PN). This result demonstrates that, although DMAP treatment is effective in oocyte activation following ICSI, the DMAP treatment can induce highly abnormal zygotes following ICSI. Therefore, further improvement of the oocyte activation procedure following ICSI is required.

Since a single Ca^{2+} stimulation resulted in a relatively low incidence of pronuclear formation and the fact that DMAP treatment could result in triploidy, activation by multiple Ca^{2+} stimulations following ICSI was tested. Oocyte activation is normally triggered by the sperm. Sperm-oocyte fusion induces a series of increases in the intracellular free Ca^{2+} concentration and the decline in histone H1 kinase activity in mammals. The pattern of Ca^{2+} release in mammalian oocytes is different among species. A large release of intracellular Ca^{2+} occurs in the hamster egg 10 to 30 sec after attachment of a fertilizing spermatozoon to the egg plasma membrane (Miyazaki *et al.*, 1986). In the mature mouse egg at fertilization, an initial large Ca^{2+} rise with a mean

duration of 2.3 min is following by several smaller rises at a 3.4 min intervals (Fissore *et al.*, 1992), and the periodic increase in Ca^{2+} persists for 1 to 3 h (Shiina *et al.*, 1993; Jones *et al.*, 1995a). Bovine oocytes exhibit a characteristic pattern both with respect to spike amplitude and spiking interval, although the general pattern of Ca^{2+} release is similar to that of other species. In mice and hamsters, the first increase in Ca^{2+} occurs immediately after insemination whereas, in cows, several hours elapse before the first Ca^{2+} increase because the time required for sperm capacitation is longer in cattle than in other species. In bovine oocytes at fertilization, multiple Ca^{2+} elevations occur in approximately 25-min intervals between spikes. Peak Ca^{2+} concentration in the first elevation is 870 ± 184*n*M, and peak Ca^{2+} concentration decreases gradually until pronuclear formation (Sun *et al.*, 1994).

Fertilization or artificial activation of oocytes results in release from meiotic arrest by inactivation of the maturation-promoting factor (MPF). Release from metaphase II is associated with loss of histone kinase activity. Fertilization triggers periodic Ca^{2+} elevations in oocytes, and these Ca^{2+} increases seem to be required for MPF inactivation. Inactivation of histone H1 kinase in oocytes allows resumption of meiosis and entry into the first embryonic cell cycle. Histone H1 kinase inactivation is dependent on extracellular Ca^{2+}, and histone H1 kinase is regulated by the number of Ca^{2+} stimulations. Ca^{2+} rises and histone H1 kinase interact at a critical point during fertilization; the Ca^{2+} response induced by the sperm during fertilization induces the decline in kinase activity that is required to exit meiosis (Chang *et al.*, 1997). Histone H1 kinase activity in fertilized oocytes decreases to 19% of its original level at 5 h after insemination, and no fluctuations in activity occur for at least 8 h (Collas *et al.*, 1993b).

5. 5. Experiment V : Pronuclear formation in bovine oocytes activated with multiple stimulations and higher concentration of Ionomycin after ICSI

5. 5. 1. Objective

To determine the effect of multiple stimulations and different concentrations of Ionomycin on activation of bovine oocytes following ICSI

5. 5. 2. Experimental design

Three Ionomycin stimulations were given at 30-min intervals. Pronuclear formation was examined at 16 h after ICSI. In addition, two concentrations of Ionomycin (5μM and 50μM) were compared on pronuclear formation in the same multiple treatment protocol, and metaphase II and III were recorded. The oocytes were fixed and then stained at 16 h.

5. 5. 3. Result and discussion

Table 7 shows development of bovine oocytes following ICSI and single or multiple treatments with Ionomycin. In untreated oocytes (Figure 10), pronuclear formation was not seen in sham-injected control group, and only 1 of 36 had one pronucleus in sperm-injected group. With multiple Ca^{2+} stimulations, pronuclear

Table 7. Development of bovine oocytes following ICSI and single or multiple treatment with Ionomycin

Group	Treatment	[a]Number of oocytes injected	Number of oocytes examined	pronuclei (%)		
				1PN	2PN	3PN
Sperm injection	untreated	46	36	1 (2.8)[c]		
	Ionomycin	46	34	5 (14.7)[b]	2 (5.9)[d]	
	multiple Ionomycin	46	41	4 (9.8)[bc]	12 (29.3)[e]	
Sham injection (control)	untreated	46	31	0 (0)[c]		
	Ionomycin	46	40	3 (7.5)[bc]		
	multiple Ionomycin	46	33	3 (9.1)[bc]		

[a] Done in six replicates

[b,c,d,e] Values with different superscripts within columns are significantly different, $p<0.05$, [d,e] $p<0.001$.

Table 8. Development of bovine oocytes following ICSI and multiple treatments with two concentrations of Ionomycin

Group	Treatment	[a]Number of oocytes injected	Number of oocytes examined	pronuclei (%)				
				[b]MII	[c]MIII	1PN	2PN	3PN
Sperm injection	untreated	23	21	19 (90.5)		1 (4.8)[de]	1 (4.8)[g]	
	5μM Ionomycin	23	23	2 (8.7)	15 (65.2)	2 (8.7)[de]	4 (17.4)[fg]	
	50μM Ionomycin	23	22	1 (4.5)	12 (54.5)	3 (13.6)[de]	6 (27.3)[f]	
Sham injection (control)	untreated	23	23	22 (95.7)	1 (4.3)	0 (0)[e]		
	5μM Ionomycin	23	23	2 (8.7)	19 (82.6)	2 (8.7)[de]		
	50μM Ionomycin	23	23	2 (8.7)	16 (69.6)	5 (21.7)[d]		

[a] Done in four replicates
[b] MII, metaphase II oocytes
[c] MIII, metaphase III oocytes
[d,e,f,g] Values with different superscripts within columns are significantly different, $p<0.05$, [f,g] $p<0.01$.

formation was observed in 16 of 41 (39.0%) oocytes at 16 h after sperm injection (Figure 11), with one and two pronuclei found in 4 of 16 (25.0%) and 12 of 16 (75.0%), respectively. There was no significant difference between single and multiple Ionomycin treatments in one pronucleus; however, there was a significant difference between the two treatments in the development of two pronuclei (5.9% vs. 29.3, $p<0.001$). In sham-injected control group treated with multiple Ca^{2+} stimulations, one pronucleus was formed in 3 of 33 (9.1%), but two pronuclei were not seen. There were no significant differences among treatments in sham-injected control group.

Due to the low rate of pronuclear formation after 5μM Ionomycin, 50μM Ionomycin was compared in the same multiple treatment protocol (Table 8).

The higher dose (50μM) resulted in a higher incidence of pronuclear formation (one and two pronuclei) when compared with the lower dose (5μM) (40.9 vs. 26.3%) after ICSI. Sham-injected oocytes activated with multiple stimulation of Ionomycin contained only one pronucleus, while sperm-injected oocytes contained one and two pronuclei, and the 50μM dose resulted in more eggs than the lower dose with two pronuclei (27.3 vs.17.4%, $p<0.05$). Both the sperm-injected and sham-control groups had high rates of metaphase III (MIII) arrested oocytes (MIII is an abnormal stage in which chromosomes remain condensed after telophase II due to an insufficient ooplasmic activation).

Based on a transient Ca^{2+} increase and histone H1 kinase decrease at fertilization, multiple stimulations with Ionomycin were given at 30-min intervals to activate bovine oocytes following ICSI. Single and multiple stimulations were not significantly different between the sperm-injected and sham-injected control groups in the formation of a single

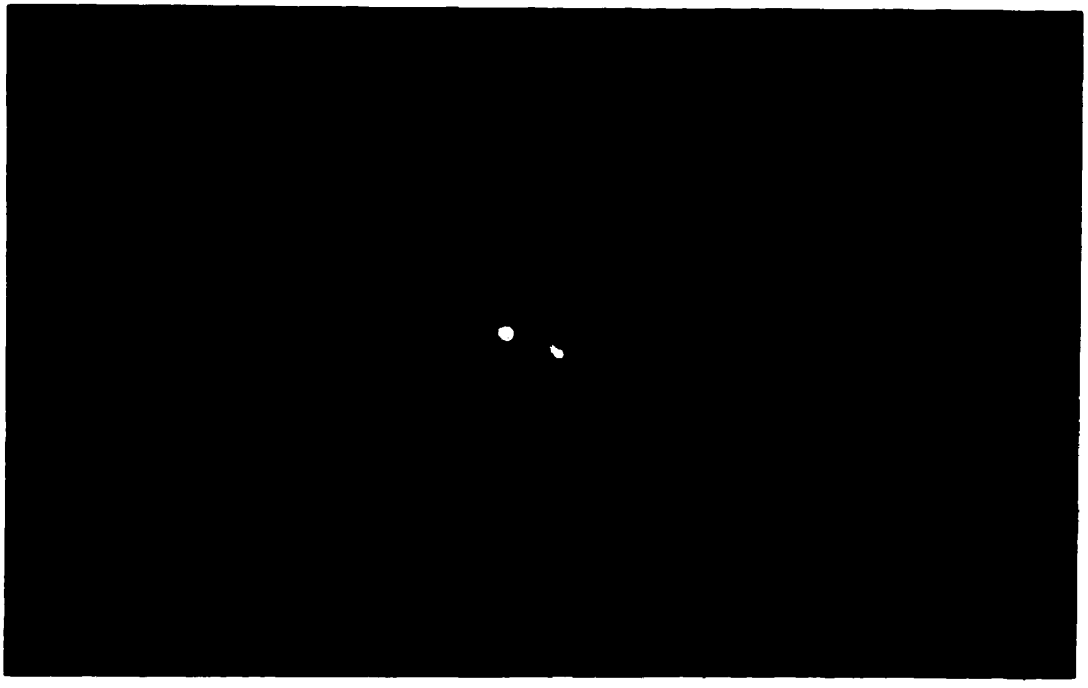


Figure 10. An untreated oocyte, 16 h after ICSI

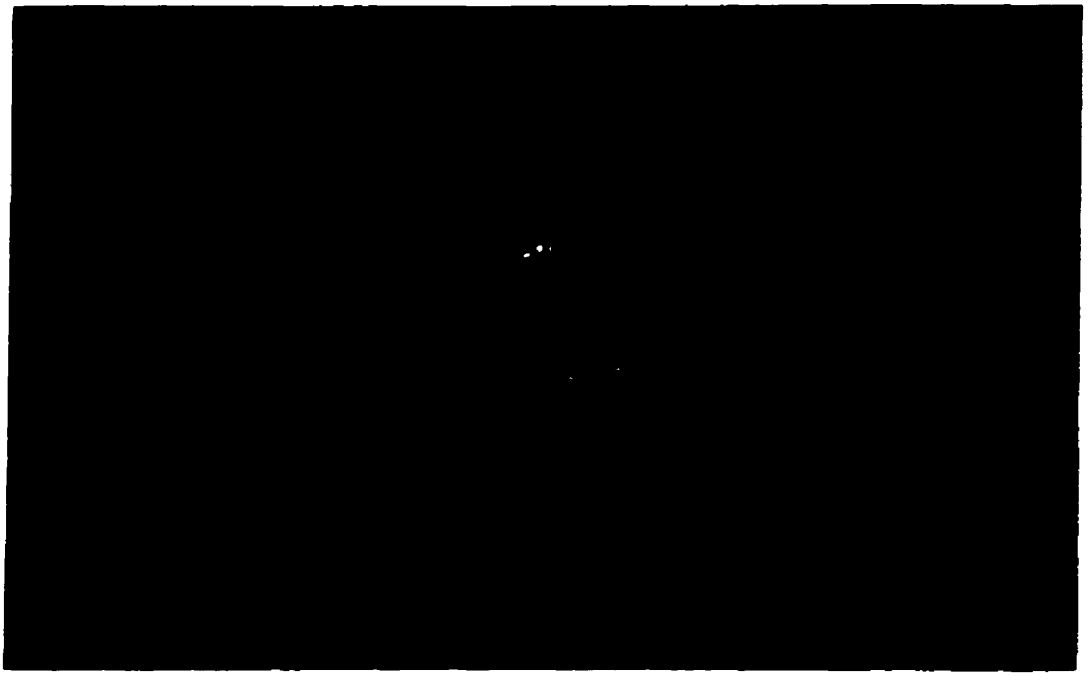


Figure 11. A pronuclear egg activated with multiple Ionomycin treatments, 16 h after ICSI

pronucleus (1PN) (14.7 vs. 9.8%, 7.5 vs. 9.1%, respectively). However, multiple stimulations induced two pronuclei in a significantly greater number of oocytes in the sperm-injected group (5.9 vs. 29.3%, $p<0.001$). This result indicates that a single stimulation is not able to activate the oocyte sufficiently to develop a spermatozoon to the pronuclear stage (male pronucleus), and although multiple stimulations result in a lower incidence of one pronucleus (probably female) than a single stimulation, multiple stimulations are more effective in the formation of normal zygotes (2PN) following ICSI. It is recognized, however, that normal (2PN) pronuclear formation is relatively low (29.3%).

The higher dose of Ionomycin (50μM) was compared in the same multiple stimulation protocol due to the low rate of pronuclear formation after 5μM Ionomycin. Pronuclear formation and metaphase III (metaphase III is an abnormal stage in which chromosomes remain condensed after telophase II due to insufficient ooplasmic activation) were recorded at 16 h after multiple stimulations following ICSI. In the sperm-injected group, most of the oocytes activated by multiple stimulations with 5μM and 50μM Ionomycin resumed the second meiotic division (91.3 vs. 95.5%, respectively), although 15 of 23 (65.2%) and 12 of 22 (54.5%) injected oocytes were arrested in metaphase III, respectively. This result indicates that, regardless of the concentration of Ionomycin, multiple stimulations can initiate oocyte activation following ICSI; however, these treatments are also insufficient to induce normal pronuclear formation. In addition, although 50μM resulted in a higher incidence of pronuclear formation (one and two pronuclei) when compared with 5μM (40.9 vs. 26.3%) after ICSI, there was no significant difference between the two treatments. The occurrence of normal

pronuclear formation (2PN) was higher following treatment with the higher dose in the sperm-injected group (27.3 vs. 17.4%, $p<0.05$), however, the percentage was still low. Therefore, further improvements are required in order to increase the rate of normal activation following ICSI in the bovine oocyte.

6. GENERAL CONCLUSIONS

1. Centrifugation of *in vitro* matured bovine oocytes is a simple and safe method that facilitates injection of the sperm into an oocyte in the ICSI procedure.

2. The ICSI technique, by itself, does not contribute to the activation of bovine oocytes.

3. The superior development of sperm-injected as compared to sham-injected oocytes suggests that the sperm contributes to the activation process.

4. Although Ionomycin and DMAP treatment results in a higher incidence of pronuclear formation, DMAP treatment can result in triploidy (abnormal).

5. A single stimulation with Ionomycin provides low activation of bovine oocytes after ICSI.

6. Multiple stimulations with Ionomycin can induce normal pronuclear formation following ICSI. The low rate of 2PN-zygote formation, however, indicates that further improvements are required in order to increase the rate of normal zygote formation following ICSI in the bovine oocyte.

7. LITERATURE CITED

Barg, P. E., Wahrman, M. Z., Talansky, B. E. and Gordon, J. W. 1986. Capacitated, acrosome reacted but immotile sperm, when microinjected under the mouse zona pellucida, will not fertilize the oocytes. J. Exp. Zool. 237:365-374.

Beil, R. E. and Graves, C. N. 1977. Nuclear decondensation of mammalian spermatozoa: changes during maturation and *in vitro* storage. J. Exp. Zool. 202:235-240.

Bellvé, A. R. 1979. The molecular biology of mammalian spermatogenesis. In "Oxford Reviews of Reproductive Biology" C. Finn, ed. Clarendon Press, Oxford, Vol. 1, pp. 159-261.

Calvin, H. I. and Bedford, J. M. 1971. Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. J. Reprod. Fertil. Suppl. 13:65-75.

Campbell, K. H. S., Ritchie, W. A. and Wilmut, I. 1993. Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. Biol. Reprod. 49:933-942.

Chang Li He, Philip Damiani, Jan B. Parys and Rafael A. Fissore. 1997. Calcium, calcium release receptors, and meiotic resumption in bovine oocytes. Biol. Reprod. 57:1245-1255.

Collas, P., Fissore, R. A., Robl, J. M. Sullivan, E. J. and Barnes F. L. 1993a. Electrically induced calcium elevation, activation, and parthenogenetic development of bovine oocytes. Mol. Reprod. Dev. 34:212-223.

Collas, P., Sullivan, E. J. and Barnes, F. L. 1993b. Histone H1 kinase activity in bovine oocytes following calcium stimulation. Mol. Reprod. Dev. 34:224-231.

Collas, P., Chang, T., Long, C. and Robl, J. M. 1995. Inactivation of histone H1 kinase by Ca^{2+} in rabbit oocytes. Mol. Reprod. Dev. 40:253-258.

Diez, C., Le Bourihis, D., Heyman, Y. and Renard, J. P. 1996. Effect of partial lipid removal from *in vitro* produced bovine zygotes on further development *in vitro* and on the freezing tolerance of blastocysts. Theriogenology 45: Abstr. 166.

Dozortsev, D., Rybouchkin, A., De Sutter, P. and Dhont, M. 1995. Sperm plasma membrane damage prior to intracytoplasmic injection: a necessary condition for sperm nucleus decondensation. Hum. Reprod. 10:2960-2964.

First, N. L., Leibfried-Rutledge, M. L., Northey, D. L. and Nuttleman, P. R. 1992. Use of *in vitro* matured oocytes 24 hr of age in bovine. Theriogenology 37: Abstr. 211.

Fissore, R. A., Dobrinsky, J. R., Balise, J. J., Duby, R. T. and Robl, J. M. 1992. Patterns of intracellular Ca^{2+} concentrations in fertilized bovine eggs. Biol. Reprod. 47:906-969.

Fissore, R. A. Gordo, A. C. and Wu, H. 1998. Activation of development in mammals: is there a role for a sperm cytosolic factor? Theriogenology 49:43-52.

Goodrowe, K. L., Wall, R. J., O'Brien, S. J., Schmidt, P. M. and Wildt, D. E. 1988. Developmental competence of domestic cat follicular oocytes after fertilization *in vitro*. Biol. Reprod. 39:355-372.

Gordon, A. C. and Carroll, M. J. 1997. Factors affecting the outcome of intracytoplasmic sperm injection (ICSI) of bovine oocytes matured *in vitro*. J. Reprod. Fertil. 19: Abstr. 64.

Gordon, J. W. and Talansky, B. E. 1986. Assisted fertilization by zona drilling: a mouse model for correction of oligospermia. J. Exp. Zool. 239:347

Goto, K., Kinoshita, A., Takuma, Y. and Ogawa, K. 1990. Fertilisation of bovine oocytes by the injection of immobilised, killed spermatozoa. Vet. Rec. 127:517-520.

Goto, K. 1993. Bovine microfertilization and embryo transfer. Mol. Reprod. Dev. 36:288-290.

Hagen, D. R., Prather, R. S. and first, N. L. 1991. Response of porcine oocytes to electrical and chemical activation during maturation *in vitro*. Mol. Reprod. Dev. 28:70-73.

Homa, S. T. 1995. Calcium and meiotic maturation of the mammalian oocytes. Mol. Reprod. Dev. 40:122-134.

Hosoi, Y., Miyake, M., Utsumi, K. and Iritani A. 1988. Development of rabbit oocytes after microinjection of spermatozoa. Proceedings of 11th International Congress on Animal Reproduction and Artificial Insemination: Abstr. 331.

Hyttel, P. and Niemann, H. 1990. Ultrastructure of porcine embryos following development *in vitro* versus *in vivo*. Mol. Reprod. Dev. 27:136-144.

Iwasaki, S., Yoshiba, N., Usijima, H., Watanabe, S. and Nakahara, T. 1990. Morphology and proportion of inner cell mass of bovine blastocysts fertilized *in vitro* and *in vivo*. J. Reprod. Fertil. 90:279-284.

Jeffe, L. F. 1983. Sources of calcium in egg activation: a review and hypothesis. Dev. Biol. 99:265-276

Jones, K. T., Carroll, J. and Whittingham, D. G. 1995a. Ionomycin, thapsigargin, ryanodine, and sperm induced Ca^{2+} release increase during meiotic maturation of mouse oocytes. J. Biol. Chem. 270:6671-6677.

Jones, K. T., Carroll, J., Merriman, J. A., Whittingham, D. G. and Kono, T. 1995b. Repetitive sperm-induced Ca^{2+} transients in mouse oocytes are cell cycle dependent. Development 121:3259-3266.

Keefer, Carol L. 1989. Fertilization by sperm injection in the rabbit. Gamete Res. 22:59-69.

Keefer, Carol L. 1990a. New techniques for assisted fertilization. Theriogenology 33:101-112

Keefer, Carol L., Younis, A. I. and Benjamin G. Brackett. 1990b. Cleavage development of bovine oocytes fertilized by sperm injection. Mol. Reprod. Dev. 25:281-285.

Keefer, Carol L. 1995. Update on bovine *in vitro* embryo production. International Embryo Transfer Society Newsletter 13 (4):8-12.

Keskintepe, L., Morton, P. C., Smith, S. E., Tucker, M. J., Simplicio, A. A. and Brackett, B. G. 1997. Carprine blastocyst development after intracytoplasmic sperm injection (ICSI). Theriogenology 47: Abstr. 249.

Kimura, Y. and Yanagimachi, R. 1995a. Intracytoplasmic sperm injection in the mouse. Biol. Reprod. 52:709-720.

Kimura, Y. and Yanagimachi, R. 1995b. Mouse oocyte injected with testicular spermatozoa or round spermatids can develop into normal offspring. Development 121:2397-2405.

Kimura, Y., Yanagimachi, R., Kuretake, S., Bortkiewicz, H., Perry, A. C. F. and Yanagimachi, H. 1998. Analysis of mouse oocyte activation suggests the involvement of sperm perinuclear material. Biol. Reprod. 58:1407-1415.

Kono, T., Iwasaki, S. and Nakahara, T. 1989. Parthenogenetic activation by electric stimulus of bovine oocytes matured *in vitro*. Theriogenology 32:569-576.

Labbe, J., Picard, A., Plaucellier, G., Cavadore, J. C., Nurse, P. and Doree, M. 1989. Purification of MPF from starfish: identification as the histone H1 kinase $p34^{cdc2}$ and a possible for its periodic activation. Cell 57:253-263.

Laws-King, A., Trounson, A., Sathananthan, A. H. and Kola, I. 1987. Fertilization of human oocytes by microinjection of a single spermatozoon under the zona pellucida. Fertil. Steril. 48:637-642.

Lim, J. M., Okitsu, O. and Niwa, K. 1994. Effects of fetal calf in culture medium on development of bovine oocytes matured and fertilized *in vitro*. Theriogenology 41:1091-1098.

Longo, F. L. 1981. Regulation of pronuclear development. In "Bioregulators of Reproduction" G. Jagiello and H. J. Vogel, eds. Academic Press, New York, pp. 529-557.

Mann, J. R. 1988. Full term development of mouse eggs fertilized by a spermatozoon microinjected under the zona pellucida. Biol. Reprod. 38:1077-1083.

Markert, C. L. 1983. Fertilization of mammalian eggs by sperm injection. J. Exp. Zool. 228:195-201.

Maro, B., Howlett, S. K. and Houliston, E. 1986. Cytoskeletal dynamics in the mouse eggs. J. Cell Sci. Suppl. 5:343-359.

Miyazaki, S., Hashimoto, N., Yoshimoto, Y., Kishimoto, T., Igusa, Y. and Hiramoto, Y. 1986. Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. Dev. Biol. 118:259-267.

Miyazaki, S. 1991. Repetitive calcium transients in hamster oocytes. Cell Calcium 12:205-216

Miyazaki, S., Shirakawa, H., Nakada, K. and Honda, Y. 1993. Essential role of inositol 1,4,5-triphosphate receptor/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization in mammalian eggs. Dev. Biol. 158:62-78.

Murray, A. W., Solomon, M. J. and Kirschner, M. W. 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature 339:280-286.

Nagai, T. 1987. Parthenogenetic activation of cattle follicular oocytes *in vitro* with ethanol. Gamete Res. 16:243-249.

Nagai, T. 1993. Development of bovine *in vitro* matured follicular oocytes activated with ethanol. Theriogenology 37:869-875.

Nagashima, H., Kashiwazaki, N., Ashman, R. J., Grupen, C. G., Seamark, R. F. and Nottle, M. B. 1994. Removal of cytoplasmic lipid enhances the tolerance of porcine embryos to chilling. Biol. Reprod. 51:618-622.

Nagashima, H., Kashiwazaki, N., Ashman, R. J., Grupen, C. G. and Nottle, M. B. 1995. Cryopreservation of porcine embryos. Nature 374:416.

Nakada, K., Mizuno, J., Shiraishi, K., Endo, K. and Miyazaki, S. 1995. Initiation, persistence, and cessation of series of intracellular Ca^{2+} responses during fertilization in bovine eggs. J. Reprod. Dev. 41:77-84.

Navara, C. S., First, N. L. and Schatten, G. 1994. Microtubule organization in the cow during fertilization, polyspermy, parthenogenesis, and nuclear transfer: the role of the sperm aster. Dev. Biol. 162:29-40.

Ng, S. C., Bongso, A., Chang, S. I., Sathananthan, H. and Ratnam, S. 1989. Transfer of human sperm into the perivitelline space of human oocytes after zona drilling or zona puncture. Fertil. Steril. 52:73-78.

Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A. C. 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 340:17-18.

Parrish, J. J., Krogenases, A. and Susko-Parrish, J. L. 1995. Effect of bovine sperm separation by either swim-up or Percoll method on success of *in vitro* fertilization and early embryonic development. Theriogenology 44:859-869.

Patel, R., Twigg, J., Crossley, I., Goldstein, R. and Whitaker, M. J. 1989. Calcium-induced chromatin condensation and cyclin phosphorylation during chromatin

condensation cycle in amonia-activated sea urchin eggs. J. Cell Biol. Suppl. 12:129-144.

Perreault, Sally D. and Barry R. Zirkin. 1982. Sperm nuclear decondensation in mammalian: role of sperm associated proteinase *in vivo*. J. Exp. Zool. 224:253-257.

Perreault, Sally D., Robert A. Wolff and Barry R. Zirkin. 1984. The role of disulfide bond reduction during mammalian sperm nuclear decondensation *in vivo*. Dev. Biol. 101:160-167.

Perreault, Sally D., Randy R. Barbee, Kenneth H. Elstein, Robert M. Zucker and Carol L. Keefer. 1988. Interspecies differences in the stability of mammalian sperm nuclei assessed *in vivo* by sperm microinjection and *in vitro* by flow cytometry. Biol. Reprod. 8:1061-1066.

Pollard, J. W. and Leibo, S. P. 1993. Comparative cryobiology of *in vitro* and *in vivo* derived bovine embryos. Theriogenology 39: Abstr. 287.

Prather, R. S., Barnes, F. L., Sims, M. M., Robl, J. M., Eyestone, W. H. and First, N. L. 1987. Nuclear transplantation in the bovine embryos: assessment of donor nuclei and recipient oocyte. Biol. Reprod. 37:857-866.

Procházka, R., Durnford, R., Fiser, P. S. and Marcus, G. J. 1993. Parthenogenetic development of activated *in vitro* matured bovine oocytes. Theriogenology 39:1025-1032.

Rho, G. J., Sheldon Kawarsky, Walter, H. Johnson, Kanwal Kochhar and Keith J. Betteridge. 1998. Sperm and oocyte treatment to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. Biol. Reprod. 59:918-924.

Rodriguez, H., Ohanian, C. and Bustos-Obregon, E. 1985. Nuclear chromatin decondensation of spermatozoa *in vitro*: a method for evaluating the fertilizing ability of ovine semen. International Journal of Andrology 8:147-158.

SAS. 1990. SAS 6.10. Statistical Analysis System institute, Inc., Cary, NC.

Sirard, M. A., Florman, H. M., Leibfried-Rutledge, M. L., Barnes, F. L., Sims, M. L. and First, N. L. 1989. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol. Reprod. 40:1257-1262.

Shiina, Y., Kaneda, M., Matsuyama, K., Tanaka, K., Hiroi, M. and Doi, K. 1993. Role of the extracellular Ca^{2+} on the intracellular Ca^{2+} changes in fertilized and activated mouse oocytes. J. Reprod. Fertil. 97:143-150.

Soloy, E., Kanaka, J., Viuff, D., Smith, S. D., Callesen, H. and Greve, T. 1997a. Time course of pronuclear deoxyribonucleic acid synthesis in parthenogenetically activated bovine oocytes. Biol. Reprod. 57:27-35.

Soloy, E., Srsen, V., Pavlok, A., Hyttel, P., Thomsen, P. D., Smith, S. D., Prochazka, R., Kubelka, M., Hoier, R., Booth, P., Motlik, J. and Greve, T. 1997b. Establishment of the block against sperm penetration in parthenogenetically activated bovine oocytes matured *in vitro*. J. Reprod. Fertil. 111:151-157.

Sun, F. Z., Hoyland, J., Huang, X., Mason, W. and Moor, R. M. 1992. A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. Development 115:947-956.

Sun, F. Z., Bradshaw, J. P., Galli, C. and Moor, R. M. 1994. Changes in intracellular calcium concentration in bovine oocytes following penetration by spermatozoa. J. Reprod. Fertil. 101:713-719.

Susko-Parrish, J. L., Leibfried-Rutledge, M. L., Northet, D. L., Schutzkus, V. and First, N. L. 1994. Inhibition of protein kinases after induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. Dev. Biol. 166:1-11.

Taylor, C. T., Lawrence, Y. M., Kingsland, C. R., Biljan, M. M. and Cuthbertson, K. S. 1993. Oscillations in intracellular free calcium induced by spermatozoa in human oocytes at fertilization. Hum. Reprod. 8:2174-2179.

Tesarik, J. and Testart, J. 1994. Treatment of sperm-injected human oocytes with Ca^{2+} ionophore supports the development of Ca^{2+} oscillations. Biol. Reprod. 51:385-391.

Tesarik, J., Sousa, M. and Mendoza, C. 1995. Sperm-induced calcium oscillations of human oocytes show distinct features in the oocyte center and periphery. Mol. Reprod. Dev. 41:257-263.

Tocharus, C., Kitiyanant, Y. and Pavasuthipaisit, K. 1996. Fertilization and subsequent development of bovine embryos after intracytoplasmic sperm injection (ICSI) using different injection pipettes. Theriogenology 45(1): Abstr. 302.

Tsien, R. W. and Tsien, R. Y. 1990. Calcium channels, stores and oscillations. Annu. Rev. Cell Biol. 6:715-760.

Uehara, T. and Yanagimachi, R. 1976. Microsurgical injection of sperm into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. Biol. Reprod. 15:467-470.

Usui, N and Yanagimachi, R. 1976. Behavior of hamster sperm nuclei incorporated into eggs at various stages of maturation, fertilization and early development. J. Ultrastruct. Res. 57:276-288.

Van Steirteghem, A. C., Nagy, Z., Joris, H., Liu, J., Staessen, C., Smitz, J., Wisanto, A. and Devroey, P. 1993. High fertilization and implantation rates after intracytoplasmic sperm injection. Hum. Reprod. 8:1061-1066.

Wall, R. J., Pursel, V. G., Hammer, R. E. and Brinster, R. L. 1985. Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei. Biol. Reprod. 32:645-651.

Wall, R. J. and Hawk, H. W. 1988. Development of centrifuged cow zygotes cultured in rabbit oviducts. J. Reprod. Fertil. 82:673-680.

Wang, W. H., Macháty, Z., Abeydeera, L. R., Prather, R. S. and Day, B. N. 1998a. Parthenogenetic activation of pig oocytes with calcium ionophore and block to sperm penetration after activation. Biol. Reprod. 58:1357-1366.

Wang, W. H., Abeydeera, L. R. and Day B. N. 1998b. Functional analysis of activation of porcine oocytes by spermatozoa, calcium ionophore and electrical pulse. Theriogenology 1998. 49: Abstr. 158.

Ware, C. B., Barnes, F. L., Maki-Laurila, M. and First, N. L. 1989. Age dependence of bovine oocyte activation. Gamete Res. 22:265-275.

Westhusin, M. E., Anderson, J. G., Harms, P. G. and Kraemer, D. C. 1984. Microinjection of spermatozoa into bovine eggs. Theriogenology 21: Abstr. 274.

Whitaker, M. J. and Patel, R. 1990. Calcium and cell cycle control. Development 108:525-542.

Yang, X., Presicee, G. A., Moraghan, L., Jiang, S. and Foot, R. H. 1994. Synergistic effect of ethanol and cycloheximide on activation of freshly matured bovine oocytes. Theriogenology 41:395-403.