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In Vitro Development of Zygotes from Prepubertal Gilts After Microinjection of DNA

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ABSTRACT: The effect of pronuclear microinjection of DNA and culture in excised mouse oviducts on the development of porcine zygotes was assessed in this study. Precocious ovulation was induced in prepubertal gilts with pregnant mare's serum gonadotrophin and hCG. Zygotes received either pronuclear microinjection of buffer alone, buffer containing a DNA construct, or no microinjection. Zygotes were cultured in vitro in either modified Krebs-Ringer bicarbonate medium (KRB) for 144 h or in mouse oviduct (MO) explant culture with KRB for 48, 72, 96, or 120 h. Pronuclear microinjection of DNA resulted in a lower ($P < .05$)

cleavage index (CI) than did buffer or no microinjection (CI $2.16 \pm .10$ vs $2.80 \pm .13$ and $2.93 \pm .10$). The CI loss was greatest for DNA-injected zygotes at the two-cell stage of development. Coculture of zygotes in MO resulted in a higher CI ($P < .01$) than did culture in KRB. Culture in MO for 72 h was the most beneficial system compared with MO for 48, 96, or 120 h ($P < .05$; CI $3.25 \pm .12$ vs $2.66 \pm .18$, $2.79 \pm .14$, and $2.40 \pm .14$, respectively). Microinjection of DNA, not merely the mechanical procedure, was detrimental to early zygote development and may be the cause of low pregnancy rates.

Key Words: Pigs, Ova Culture, Transgenics

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Introduction

Research directed toward the production of transgenic animals is hampered by the inadequate in vitro development of zygotes. Petters et al. (1990) found 45 to 60% morula and blastocyst development from one- and two-cell porcine embryos cultured in Krebs-Ringer bicarbonate buffer (KRB) modified with glucose and glutamine. Others have reported comparable success with a modified Tyrode's medium supplemented with glucose and

glutamine (Hagen et al., 1991). However, other studies have shown that the use of media supplemented with porcine oviductal epithelial cells results in a greater proportion of two-cell porcine embryos developing to blastocyst than those in medium alone (62 vs 0%; White et al., 1989). Krisher et al. (1989) used in vitro coculture of porcine zygotes for 6 d in explanted mouse oviducts. With this procedure, 78% of the zygotes reached the morula or blastocyst stage compared with 36% without oviducts. Although complex systems allow for increased in vitro development of one-cell zygotes, the optimal incubation times have not been established.

Our objective was to evaluate culture procedures and incubation times for zygote development after DNA microinjection.

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Materials and Methods

Precocious ovulation was stimulated in 40 prepubertal gilts 132 to 197 d of age. These gilts were classified as prepubertal by lack of observed estrus, age, weight, appearance of external genitalia, and herd history. Ovulation was synchronized by administration of 1,500 IU of pregnant mare's serum gonadotrophin (PMSG; Diosynth, Chicago, IL) followed by 500 IU of hCG (chorionic gonadotrophin, Lyphomed, Rosemont, IL; Pinkert et al., 1989) 68 h later. Gilts were bred by AI at 24 and 36 h after the injection of hCG followed by laparotomy 52 to 57 h after the injection.

Ovarian Observation and Zygote Collection. Surgical anesthesia was initiated with 1.5 g of Pentothal® (Thiopental sodium, Abbott Laboratories, N. Chicago, IL) and maintained with halothane and nitrous oxide. Ovarian examination was accomplished after midventral laparotomy. The number of ovulations (corpora hemorrhagica, CH) and unovulated follicles by diameter were recorded for each ovary. The oviducts of animals serving as donors were flushed by inserting a silastic cannula through the ostium of the infundibulum that reached approximately 6 to 8 cm into the ampulla of the oviduct. A two-piece collection cannula constructed of 1.98 mm i.d., 3.18 mm o.d. tubing (Silastic®, Dow Corning, Midland, MI) with a heat-flared funnel of 1.57 mm i.d., 2.08 mm o.d. polyethylene tubing (Intramedic®, Clay Adams, Parsippany, NJ) fitted to the inserted end of the larger tubing was used to flush the oviducts. Two 10-mL volumes of PBS (Embryo Transfer Freezing Medium, Gibco, Grand Island, NY) supplemented with .4% albumin, 10% newborn calf serum, and .025 g/L of antibiotic (Kanamycin Sulfate, Gibco; PBS1) were flushed through the oviduct. The flushing fluid was collected into sterile test tubes and transported to the laboratory at 37°C.

Microinjection of DNA Construct. Zygotes were held in PBS1 at 37°C during all manipulations. To allow for visualization of pronuclei, zygotes were centrifuged at 15,000 × *g* for 8 min (Wall et al., 1985). After centrifugation, zygotes were examined for the presence of accessory sperm and pronuclear formation using Hoffman modulation optics (200×). One-cell structures with no visible pronuclei were classified as unfertilized ova (UFO). Zygotes, as determined by presence of pronuclei, were randomly assigned to treatments of either no injection, buffer injection, or DNA injection. Buffer and DNA microinjection of zygotes was accomplished by inserting a glass injection pipette (outside tip diameter 1.0 to 1.5 μm) into the most visible pronucleus while the zygote was held stationary with suction from a blunt, polished holding pipette. One to two picoliters of buffer or

DNA construct was delivered using an automated microinjector (Model 5242; Eppendorf, Freemont, CA).

The DNA used for microinjection was a 9.3-kb (kilobase) fragment consisting of a genomic clone for the mouse whey acidic protein (WAP) gene (Andres et al., 1987) with the human protein C (PC) cDNA inserted into the *KpnI* site of the first exon. The WAP PC construct was purified from the plasmid vector using HPLC (Velandar et al., 1992). The DNA was in 10 mM Tris-HCl, .25 mM EDTA (pH 7.4) buffer at a concentration of 5 μg/mL (~500 copies/pL).

Zygote Culture. Zygotes recovered from prepubertal gilts were used in each of two replicates to evaluate the development in mouse oviduct (MO) organ culture. Oviducts were collected from immature CD1 (21- to 28-d-old) mice (Charles River Laboratories, Wilmington, MA) that were superovulated by injection of 7 IU of PMSG followed by injection of 7 IU of hCG 48 h later. Each female was placed with one intact male at the time of hCG injection. Mice that exhibited a vaginal plug the following morning were used. Oviducts were removed 20 to 22 h after the injection of hCG and prepared as described by Krisher et al. (1989). Zygotes that were either not injected, buffer-injected, or DNA-injected were transferred into mouse oviducts using a fine glass pipette or were maintained as controls in modified KRB medium (Davis and Day, 1978) without lactate and pyruvate (Krisher et al., 1989). Three to thirteen zygotes were transferred into the swollen ampullary region of each oviduct. Oviducts were placed on a raft of .20-μm filter paper (Tuffryn® Membrane filter, Gelman Sciences, Ann Arbor, MI) suspended in KRB. The organ culture was held at 37.5°C in a humidified atmosphere of 5% CO₂ in air. Mouse embryos within the oviduct were used to assess viability of the organ culture. Porcine ova classified as UFO were not microinjected but were cultured in KRB.

Porcine zygotes within MO were removed after either 48, 72, 96, or 120 h of culture and placed in KRB for the remainder of the culture period. These treatments were identified as MO 48, 72, 96, and 120, respectively. Development of zygotes cultured in KRB was visually assessed at 24-h intervals, whereas zygotes cultured in MO were assessed upon termination of organ culture and at 24-h intervals thereafter. Normal development was assumed when embryos did not fragment or remain static at one developmental stage. Duration of culture was 144 h (Krisher et al., 1989). Embryo development was scored using a cleavage index (CI) in which two-cell embryos equal CI of 1, 3- to 4-cell = 2, 5- to 8-cell = 3, 9- to 16-cell = 4, morula = 5, and blastocyst = 6.

Statistical Analysis. Variation in development of cultured zygotes was tested by two methods. Chi-square analysis was used to determine differences in the distribution of embryo CI due to microinjection or culture treatments. Least squares means and analysis of variance in CI within treatments were determined by the GLM procedure and were tested against the *F*-statistic. Nonorthogonal contrasts, tested against the improved Bonferroni *F* statistic, were used to compare differences between treatments (SAS, 1985).

Results

Pronuclear Microinjection. Four hundred thirty-six (72.9%) of 598 pronuclear microinjected zygotes that were cultured in vitro cleaved. Neither pronuclear microinjection nor culture method affected the proportion of zygotes that cleaved to two-cells ($P > .05$). Only cultured zygotes that achieved a CI ≥ 1 were included in further statistical analysis of maximum CI. Zygotes that were initially classified as UFO and attained a CI of 1 were considered nonmicroinjected zygotes cultured in KRB. Analysis of CI excluding UFO was similar to using them in the data set. A total of 514 zygotes with a CI ≥ 1 was included in the final data set.

Microinjection affected ($P < .01$) maximum CI attained in vitro. At 144 h, maximum CI for noninjected, buffer-, or DNA-microinjected embryos was $2.93 \pm .10$, $2.80 \pm .13$, and $2.16 \pm .10$, respectively ($\bar{x} \pm \text{SEM}$, Figure 1). The DNA-injected group had a lower CI ($P < .01$) than the buffer-injected group, whereas the noninjected group had a greater CI ($P < .01$) than the DNA injection treatment but not a greater CI than the buffer group. However, the effect of DNA microinjection was manifested by decreased development past the two-cell stage (Table 1).

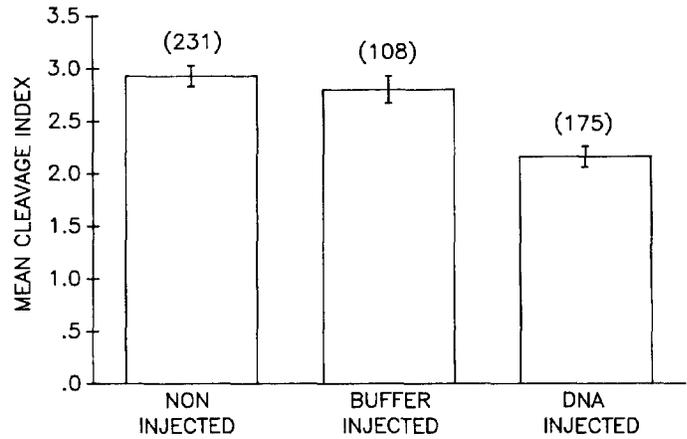


Figure 1. Mean cleavage indices attained by zygotes receiving different microinjection treatments after 144 h in culture. Cleavage index 1 = 2-cell, 2 = 3- to 4-cell, 3 = 5- to 8-cell, 4 = 9- to 16-cell, 5 morula, and 6 = blastocyst. SEM are indicated by the vertical bars (n).

Culture System. Maximum CI differed ($P < .01$) at 144 h. In vitro culture of zygotes in excised MO for 72 h resulted in the highest CI ($3.25 \pm .12$), whereas maintaining zygotes in KRB produced the lowest CI ($2.04 \pm .09$; Figure 2). Culture in excised MO for 48, 96, or 120 h resulted in CI of $2.66 \pm .18$, $2.79 \pm .14$, and $2.40 \pm .14$, respectively. Nonorthogonal contrasts showed that overall CI attained in KRB ($2.04 \pm .09$) was lower ($P < .01$) than the CI in MO ($2.77 \pm .07$). The CI for MO 72 was greater ($P < .05$) than that for MO 48, 96, and 120 ($P < .05$). The interaction ($P < .01$) of microinjection \times time in oviduct culture showed that maximum CI was attained at 72 h for noninjected and DNA-injected zygotes, whereas buffer-injected zygote development was greatest for 96 h in MO (Figure 3). At the end of each explant culture period, 79% of mouse

Table 1. Distribution of porcine zygotes obtained from superovulation of prepubertal gilts reaching specific developmental stages after 144 hours of in vitro culture in Krebs-Ringer Bicarbonate (KRB) buffer or mouse oviducts explanted in KRB^a

Maximum development	CI ^b	Non-injected	Buffer-injected	DNA-injected
2-cell	1	10.4 (24) ^c	8.3 (9)	30.9 (54)
3- to 4-cell	2	47.6 (110)	41.7 (45)	40.6 (71)
5- to 8-cell	3	26.6 (62)	36.1 (39)	20.0 (35)
9- to 16-cell	4	3.6 (13)	.9 (1)	3.4 (6)
Morulae	5	2.2 (5)	4.6 (5)	.6 (1)
Blastocysts	6	7.4 (17)	8.3 (9)	4.6 (8)
Total	—	(231)	(108)	(175)

^aChi-square $P < .01$ between treatments.

^bCleavage index.

^cPercentage and (number).

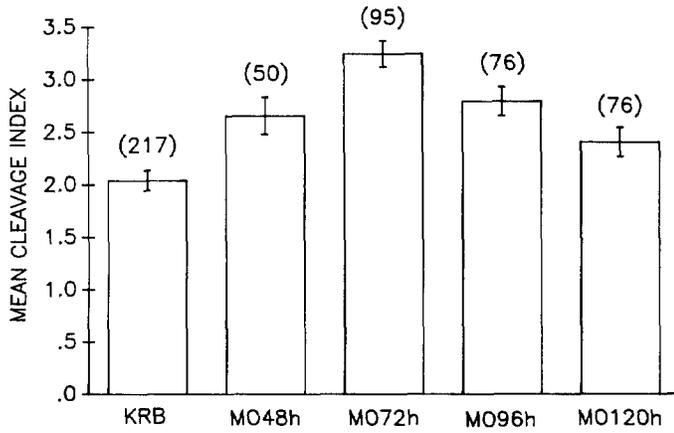


Figure 2. Mean cleavage indices attained by zygotes after 144 h in different culture systems. Legend abbreviations are as follows: KRB is Krebs-Ringer bicarbonate buffer, MO48h is 48 h in mouse oviduct culture, MO72h is 72 h in mouse oviduct culture, MO96h is 96 h in mouse oviduct culture, and MO120h is 120 h in mouse oviduct culture. Embryos were removed from mouse oviducts at the indicated times and further cultured in KRB alone for the duration of the culture. SEM are indicated by the vertical bars (n).

oviducts had normal, stage-specific, developing mouse embryos (e.g., mouse embryos were four-cell at MO 48).

Discussion

The observation that injection of DNA impaired development compared with buffer injection or no injection suggests either that DNA, not merely the mechanical procedure of pronuclear injection, was altering the pronuclear environment or that the DNA construct was being integrated into vital regions of the genome in such a way that development was inhibited. It is possible that chemical and physical contamination of the DNA could contribute components that could be detrimental to development. Hammer et al. (1986) reported that DNA microinjection decreased zygote survival to the blastocyst stage by approximately 50% in both sheep and swine compared with noninjected controls. Robl and First (1985) found that development of pronuclear microinjected mouse zygotes to the morula or blastocyst stage was not affected by DNA injection, buffer injection, or by inserting the tip of the pipette into the pronucleus with no injection. However, development was less than in those that experienced no manipulation. The exact mechanism(s) by which DNA microinjection causes embryonic death is not known. Surprisingly, the microinjection event alone did not inhibit *in vitro* develop-

ment of porcine zygotes. These results contrast with work reported in mice (Brinster et al., 1985) that injection of buffer alone decreases *in vitro* embryo development similar to DNA injection, suggesting that porcine zygotes are more resilient than mouse zygotes.

Bavister (1988) noted that in non-rodent species, developmental block of embryos maintained *in vitro* tended to occur at the stage at which the embryos move out of the oviduct and into the uterus *in vivo*. Our data show that the culture of porcine zygotes in the oviduct of a mouse for as little as 48 h allowed development past the four-cell block, which was not attained by culture in KRB alone. However, coculture within MO for 72 h was necessary for maximal zygote development. Development of zygotes held in MO for 96 and 120 h was lower than that of zygotes held in MO for 72 h, suggesting that the oviduct culture environment was not adequate for later stages of porcine embryo development. The lower zygote development could be due to an inappropriate environment present in MO that cannot support porcine embryos. This may also be indicative of decreased viability of oviductal cells in organ culture. Dying cells would release substances that could adversely affect embryo viability. The percentage of morula and blastocyst development (22%) for our noninjected prepubertal zygotes was lower than

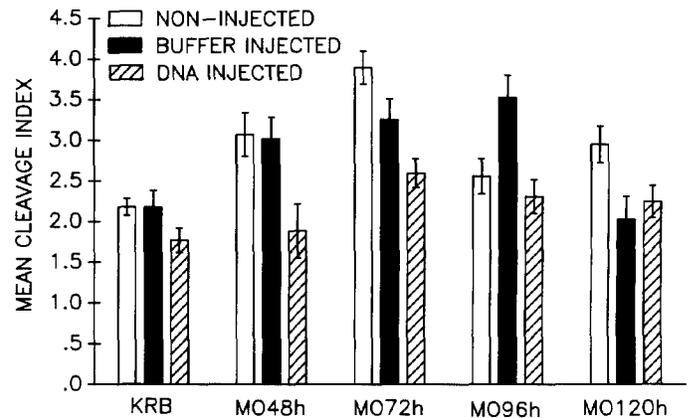


Figure 3. Mean cleavage indices for zygotes after 144 h in culture that were untreated or injected with buffer or DNA. Microinjection by mouse oviduct interaction was significant ($P < .01$). Legend abbreviations are as follows: KRB is Krebs-Ringer bicarbonate buffer, MO48h is 48 h in mouse oviduct culture, MO72h is 72 h in mouse oviduct culture, MO96h is 96 h in mouse oviduct culture, and MO120h is 120 h in mouse oviduct culture. Embryos were removed from mouse oviducts at the indicated times and further cultured in KRB alone for the duration of the culture. SEM are indicated by the vertical bars.

the 78% reported by Krisher et al. (1989) using the same MO culture system and lower than the 45 to 60% morula and blastocyst development in a more recent report by Petters et al. (1990). Both of these studies used one- and two-cell porcine embryos obtained from sows that were not synchronized or superovulated. The lower embryonic developmental rate may be related to differences in follicular development, which may affect subsequent embryonic development. Wiesak et al. (1990) reported a dissimilar pattern of follicular development in PMSG- and hCG-treated, immature gilts compared with naturally cycling pigs. Also, porcine zygote development *in vitro* was greater when zygotes were collected from mature gilts than when they were collected from prepubertal gilts (Pinkert et al., 1989).

Implications

Maintaining porcine zygotes within explanted mouse oviducts for as little as 48 h allows significantly improved development compared with culture in medium alone. When considering the detrimental effect of pronuclear microinjection, the impairment to development was found to be caused by the injection of DNA and(or) possible contaminants and not by the mechanical process of microinjection itself.

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