

Bovine Follicular Dynamics, Oocyte Recovery, and Development of Oocytes Microinjected with a Green Fluorescent Protein Construct

M. S. CHAUHAN,* S. NADIR,† T. L. BAILEY,†
A. W. PRYOR,* S. P. BUTLER,* D. R. NOTTER,‡
W. H. VELANDER,§ and F. C. GWAZDAUSKAS*,1

*Department of Dairy Science, †Large Animal Clinical Sciences,
‡Animal and Poultry Sciences, and §Chemical Engineering,
Virginia Polytechnic Institute and State University, Blacksburg 24061-0315

ABSTRACT

The present study was carried out to 1) evaluate the viability of in vitro fertilized zygotes after microinjection of DNA, 2) assess the influence of oocyte quality upon the development rate of embryos when injected with DNA, and 3) determine the integration frequency of green fluorescent protein DNA into microinjected embryos. Oocytes were aspirated from ovaries of nine nonlactating Holsteins and were categorized into grades A, B, C, and D. At 16 h after in vitro fertilization, approximately half of the pronuclear stage presumptive zygotes were classified as having 1 pronucleus or 2 pronuclei, and they were microinjected with DNA constructs. A potential predictor of DNA integration frequency at d 10 was assessment of the incidence of green fluorescing embryos. The proportion of cleaved embryos that developed to morulae or blastocysts was not different between groups with 1 pronucleus injected (45%), 1 pronucleus uninjected (64%), or 2 pronuclei injected (49%). However, the development of morulae or blastocysts was higher in the group with 2 pronuclei uninjected (69%). The overall developmental score of green fluorescent protein-positive embryos was higher for grade A oocytes (1.3 ± 0.1) than for grade B (0.8 ± 0.1), C (0.6 ± 0.1), or D (0.3 ± 0.1) oocytes. The results show that production of transgenic bovine blastocysts can occur from the microinjection of a presumptive zygote having only one visible pronucleus. Initial oocyte quality is an important factor in selection of oocytes suitable for microinjection of DNA and for preimplantation development to produce bovine transgenic embryos.

(**Key words:** bovine oocytes, in vitro fertilization, ovum pickup, green fluorescent protein)

Abbreviation key: CMV-EGFP = cytomegalovirus enhanced green fluorescent protein, GFP = green fluorescent protein, IVF = in vitro fertilization, IVM = in vitro maturation, PCR = polymerase chain reaction, 0PN = no pronucleus, 1PN = 1 pronucleus, 2PN = 2 pronuclei, WAP6FIX = whey acidic protein factor IX.

INTRODUCTION

Production of transgenic cattle through in vitro maturation (IVM) and in vitro fertilization (IVF) requires large numbers of oocytes, which are used for pronuclear microinjection of DNA. These oocytes can be obtained either from slaughter house ovaries or harvested from live animals through ovum pickup. By use of bovine IVM-IVF systems, only 60 to 80% of good quality oocytes are fertilized and 30 to 40% develop to the blastocyst stage (28, 34). Ovum pickup techniques yield a substantial number of poor quality oocytes (50 to 55%) harvested along with the good quality oocytes (14, 15). More than half of the poor quality oocytes are without cumulus cells. Association of cumulus cells with the oocyte is important for proper cytoplasmic maturation, pronuclear formation, and subsequent development (7, 9, 28, 36).

Many years of research efforts have been devoted to increase the success of transgenic animal production. The polymerase chain reaction (PCR) was used to detect the transgene before embryo transfer to a recipient to decrease the time and production costs by screening embryos (19, 20, 23). However, PCR analysis only confirms the presence of microinjected DNA, regardless of its integration status. A simple and reliable method is required to distinguish between nonintegrated and integrated DNA in microinjected preimplantation embryos. Recently, green fluorescent protein (GFP) was used as a marker of gene expression and a fusion tag to monitor protein localization within living cells (8, 10). Green fluorescent protein

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¹Correspondence and reprint requests.

appears not to have any detrimental effect on the viability of mouse embryos (17, 29). Moreover, this protein does not require any substrate for detection and may be a suitable marker for the selection of transgenic bovine embryos (30).

In bovine species, a major problem in the production of a transgenic animal is the lack of substantial numbers of good quality oocytes that can be utilized for microinjection of DNA and subsequent *in vitro* culture to the preimplantation stage prior to transfer. To increase the number of oocytes available for microinjection, developmental competence of various grades of oocytes needs to be evaluated. Therefore, the purpose of the present study was to: 1) evaluate the viability of the presumptive zygote with only one visualized pronucleus microinjected with DNA after IVF, 2) assess the influence of oocyte quality upon cleavage and development rates of embryos when injected with DNA, and 3) determine the integration frequency of GFP DNA into microinjected embryos.

MATERIALS AND METHODS

Culture Media

All chemicals and media were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated. Medium for ovum pickup was Dulbecco's PBS (Gibco, Grand Island, NY) supplemented with 0.3% BSA and 10 μg of heparin/ml (H-3145). The medium used for *in vitro* oocyte maturation was 25 mM HEPES-buffered tissue culture medium-199 supplemented with 10% (vol/vol) fetal bovine serum, 0.2 mM sodium pyruvate, 0.02 U FSH (F-2293), and 1 μg of estradiol-17 β /ml. The medium for *in vitro* fertilization was the Brackett-Oliphant medium (5) containing 10 μg of heparin/ml, 2.5 mM caffeine (C-0750), and 0.5% fatty acid-free BSA (A-8806). The *in vitro* culture medium for the first 6 d was modified synthetic oviductal fluid medium (35) supplemented with 0.3% BSA; for the next 4 d, the medium was changed to 25 mM HEPES-buffered tissue culture medium-199 supplemented with 5% fetal bovine serum and 0.1% BSA. All media were fortified with 50 μg of gentamicin/ml. All culture conditions were maintained at 38.5°C in humidified air with 5% CO₂.

Ovum Pickup Procedures

Oocytes were harvested from nine nonlactating Holstein cows twice weekly (Tuesday and Friday) for 12 wk using transvaginal follicular aspiration techniques as described by Gibbons et al. (14). Briefly,

ovum pickup was performed by manipulating the ovaries per rectum while puncturing the follicles (≥ 2 mm) with a 17-ga, 55-cm needle with an echogenic tip (RAM Consulting, Madison, WI). The ovaries were visualized with a sector ultrasound transducer (5 MHz) packaged in a vaginal probe equipped with a dorsal-mounted needle guide. The diameters (category: <6, 6 to 8, and >8 mm) of all the visualized follicles were recorded, and total numbers were counted. The follicles were pierced with the needle, and the follicular contents were removed under vacuum (<75 mm Hg) and aspirated through teflon tubing in PBS supplemented with 0.3% BSA and 10 μg of heparin/ml. The oocytes were visualized and graded under a stereomicroscope and transferred into 5 ml of 25 mM HEPES-buffered tissue culture medium-199 supplemented with 10% fetal bovine serum.

Oocyte Quality Evaluation and *In Vitro* Maturation

Bovine oocytes were evaluated and classified into four groups according to the grading system of De Loos et al. (11). Briefly, Grade A oocytes had compact, multilayered cumulus cells and homogeneously organized ooplasm; Grade B had compact cumulus cells with homogeneously organized ooplasm; Grade C oocytes had less compact cumulus cells with irregular ooplasm containing dark clusters in the ooplasm; and Grade D oocytes were without cumulus cells or had over-expanded cumulus cells and a jelly-like matrix. After collection, oocytes were washed 3 times with 25 mM HEPES-buffered tissue culture medium-199 supplemented with 10% fetal bovine serum and were cultured in IVM medium in separate groups by grade in 50- μl drops (5 to 10 oocytes per drop) covered with mineral oil for 24 h. After 24 h of maturation, the oocytes were washed separately three times with IVF medium and were incubated in IVF medium in 50- μl drops (5 to 10 oocytes per drop) until the sperm were added.

Sperm Preparation and IVF

The spermatozoa used for IVF were from one ejaculate and had been tested earlier (19). Two straws of frozen-thawed ejaculated semen were washed twice by centrifugation at 500 $\times g$ for 5 min with Brackett-Oliphant medium (5) containing 10 μg of heparin/ml but without BSA or caffeine. The pellet obtained was resuspended in the IVF medium, and the sperm numbers were adjusted at a concentration of 4 to 5 million cells/ml. Fifty microliters of sperm were added to each

50- μ l drop of IVF medium containing matured oocytes. The final concentration of sperm was approximately 0.25 million sperm/drop.

Microinjection of DNA

At 16 h of coincubation with spermatozoa, the oocytes were stripped of cumulus cells by vortexing in glucose-free TL-HEPES and were centrifuged at $13,000 \times g$ for 13 min to visualize the pronuclei. Microinjection of DNA (1 to 5 pl of DNA solution containing a 5- μ g/ml concentration) was performed in TL-HEPES as described by Gibbons et al. (15). Whey acidic protein-Factor IX (**WAP6FIX**) and cytomegalovirus-enhanced green fluorescent protein (**CMV-EGFP**) DNA constructs were microinjected separately.

The transgene construct WAP6FIX was produced by inserting a modified Factor IX cDNA sequence between a murine whey acidic protein promoter and its corresponding 3' untranslated region. The 4.1 kbp promoter was obtained by digestion of p227.6 (Henryk Lubon, American Red Cross, Rockville, MD) with *NotI*, *KpnI*, and *HindIII* with resulting fragments separated on a 0.8% agarose gel. The promoter fragment was excised from the gel and purified using a Qiagen gel extraction kit (Qiagen Inc., Chatsworth, CA). The fragment containing the 3'UTR and pUC *NotI* + vector was obtained by partial digestion of pUCWAP5 with *NotI*, and a full digestion with *KpnI* and was similarly purified. The resulting fragments were then ligated with T4 DNA ligase (Stratagene, LaJolla, CA) to make pUCWAP6, and the mixture was used to transform *Escherichia coli* JM109 cells (Stratagene) per the manufacturer's instructions. The Factor IX cDNA used in WAP6FIX was modified by adding *KpnI* restriction enzyme sites directly before the start sequence and after the stop sequence by PCR. Reaction conditions for PCR were in a 100- μ l total volume with 30 ng of plasmid template (pMCDSFIX: obtained from Darryl Stafford; University of North Carolina, Chapel Hill, NC), 0.5 μ M of each primer (hFIX5' *KpnI*, 5'GCTAGGTACCATGCAGCGCG and FIX 3' *KpnI*, 5'GTCAGGTACCTT-AAGTGAGCT), 200 μ M dNTP, 2.5 U of PFU DNA polymerase, and IX buffer, with the reaction mixture subjected to 30 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 1 min, and elongation at 75°C for 5 min 45 s. After cycling, the product was purified and cut with *KpnI*. The resulting fragment was cloned into pUC18 (Stratagene), and the insert was subjected to sequencing. The modified FIX cDNA was cut with *KpnI* and ligated into the *KpnI* site of pUCWAP6

to make pUCWAP6FIX. The resulting mixture was used to transform *E. coli* JM109 cells (Stratagene). Plasmid was purified from harvested cells by column absorption (Maxi prep; Qiagen Inc.). The linear DNA construct was excised with *NotI*, separated from plasmid by agarose gel electrophoresis, purified by silicon bead absorption (Ultrasclean 15, MoBio, Encinitas, CA), and diluted to 5 μ g/ml in 10 mM Tris and 0.25 mM EDTA at pH 7.4 for microinjection.

The transgene construct CMV-EGFP was produced by removing the inter-ribosome entry site of pIRES-EGFP (Clontech Laboratories; Palo Alto, CA) by *BamHI* endonuclease digestion. The restriction products were separated, and the vector-containing fragment was subjected to ligation. Ligation products were used to transform *E. coli* XL-Blue (Stratagene) with selection on ampicillin plates. The plasmid was isolated and purified by column absorption (Maxi prep; Qiagen Inc.). The linear DNA construct was excised with *NruI* and *XhoI*, separated from the plasmid by agarose gel electrophoresis, purified by silicon bead absorption (Ultrasclean 15, MoBio), and diluted to 5 μ g/ml in 10 mM Tris and 0.25 mM EDTA at pH 7.4 for microinjection.

Pronuclei were visualized at 16 h after insemination under the Hoffman modulation contrast optics (200 \times) in all four grades of oocytes. The pronuclear stage of the zygote was classified into 3 categories based on the number of visualized pronuclei. Zygotes were separated based on visualization of 1 pronucleus (**1PN**), 2 pronuclei (**2PN**), and without any visualized pronucleus (**0PN**). Zygotes with 1PN and 2PN of each category were randomly divided into two groups, the control and treatment (injected). Zygotes visualized with ≥ 3 pronuclei were discarded.

In Vitro Culture of Embryos

All of the injected (treatment) and uninjected (control) zygotes in each Grade and pronuclear category were cultured in a 20- μ l drop (1 to 4 zygotes per drop) of in vitro culture medium for 10 d. On d 2 (42 to 44 h) and d 10 after insemination, the cleavage rate and development of morulae and blastocysts were recorded. The expression of GFP was evaluated on d 10 using a fluorescent microscope with excitation at 488 nm and detection at 500 to 530 nm. The GFP integration was confirmed by observation of green cells (GFP positive). The 0PN oocytes were also cultured in in vitro culture medium to determine whether late fertilization occurred and to evaluate subsequent development.

Statistical Analysis

Data were analyzed by ANOVA and chi-square using the general linear model and CATMOD procedures in SAS (26). Analysis of variance models were used to evaluate effects of cow, aspirator, and time on oocyte quality, number of oocytes recovered, number of follicles observed, and follicle size. A development score for each stage of embryo development (1 = 1- to 2-cell, 2 = 3- to 8-cell, 3 = 9- to 16-cell, 4 = morula, and 5 = blastocyst) was analyzed by ANOVA with grade, treatment (pronuclear classification), and their interaction as independent variables. Chi-square models involved grade by development stage and treatment by development stage effects; CATMOD analyses were performed to compliment these chi-square analyses.

RESULTS

Follicular Dynamics and Oocyte Recovery

The mean number of follicles (13.9 ± 4.6 /cow per aspiration) varied significantly ($P < 0.01$) among cows. The number of follicles observed increased linearly from wk 1 to 12 of aspiration (Figure 1). The number of follicles with a diameter of 6 to 8 mm or <6 mm increased linearly ($P < 0.05$) from the beginning to the end of the study (Figure 2). The mean number of follicles with a diameter of 6 to 8 mm and <6 mm per cow per session was 2.0 ± 1.6 and 10.8 ± 4.1 , respectively. The number of follicles with diameters <6 mm varied significantly among the cows ($P < 0.05$). The number of follicles >8 mm did not vary throughout the aspiration period and averaged 1.1 ± 1.0 per cow per session.

The mean number of oocytes recovered per aspiration (6.5 ± 3.9) decreased linearly as the aspiration trial progressed (Figure 3). The mean number of oocytes recovered by grades was maximum for Grade D (2.3 ± 1.9) followed by C (2.0 ± 1.5), B (1.2 ± 1.1), and A (0.9 ± 1.1). Only the recovery of Grade B oocytes was affected ($P < 0.05$) by the aspiration session, and the number of Grade B oocytes declined linearly (Figure 3). However, aspiration session had no significant affect ($P > 0.05$) on the quality of Grade A, C, or D oocytes recovered.

Microinjection of Zygotes

A total of 1150 oocytes was evaluated to assess pronuclear formation at 16 h after insemination. Three hundred twenty-nine (28.6%) presumptive zygotes were visualized with 1PN, 271 (23.5%) with

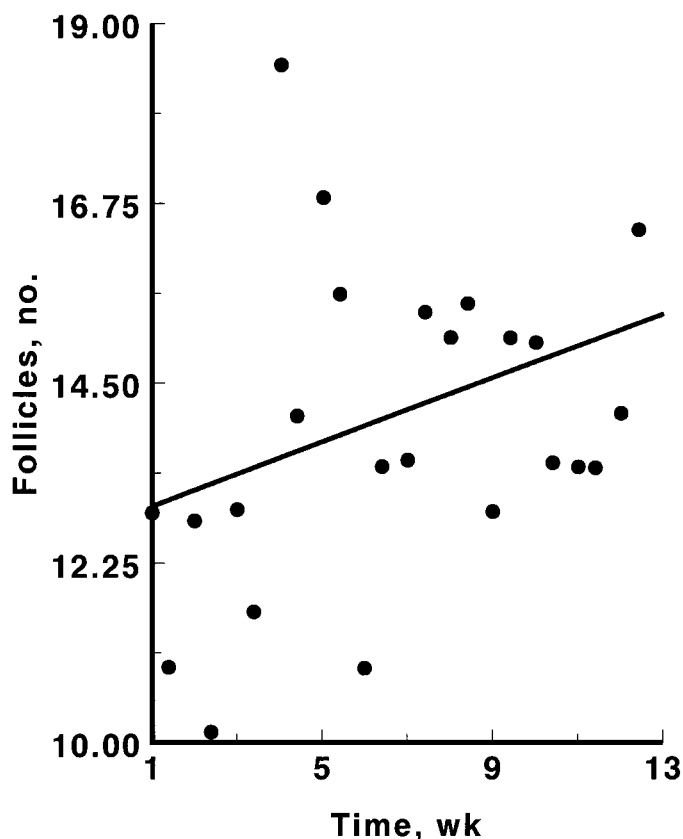


Figure 1. Linear increase in total number of follicles through 12 wk of ovum pickup in nonlactating dairy cows with twice weekly aspiration (●, session mean).

2PN, 510 (44.3%) with 0PN, and 40 (3.4%) had more than 2PN. The zygotes having more than 2PN were considered polyspermic and were not included in the study. The cleavage rate, proportion of cleaved embryos that developed to the morula or blastocyst stage, and proportion of embryos that arrested at 2- to 16-cell stage for injected and uninjected presumptive zygotes are shown in Table 1. The overall cleavage rate was significantly higher ($P < 0.01$) in the uninjected control than in the injected groups. A significant difference ($P < 0.01$) was observed in the cleavage rate between the uninjected and injected presumptive zygotes having 1PN. Similarly, there was a difference ($P < 0.01$) between the uninjected and injected groups of zygotes having 2PN. However, no significant differences ($P > 0.05$) were observed between the cleavage rate of the 1PN uninjected and 2PN uninjected zygotes or between 1PN injected and 2PN injected zygotes. Only 19% of presumptive zygotes with no visible pronuclei were cleaved; this was a significantly ($P < 0.01$) lower rate compared with other groups. The proportion of cleaved embryos

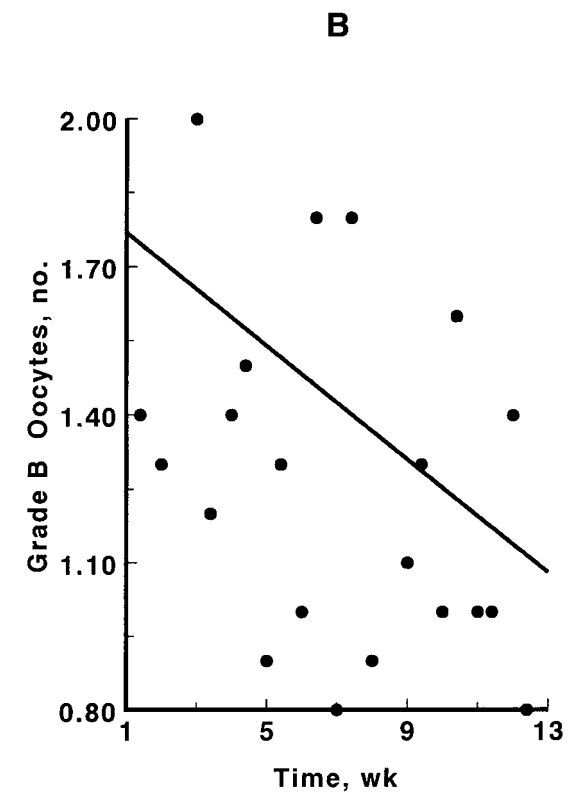
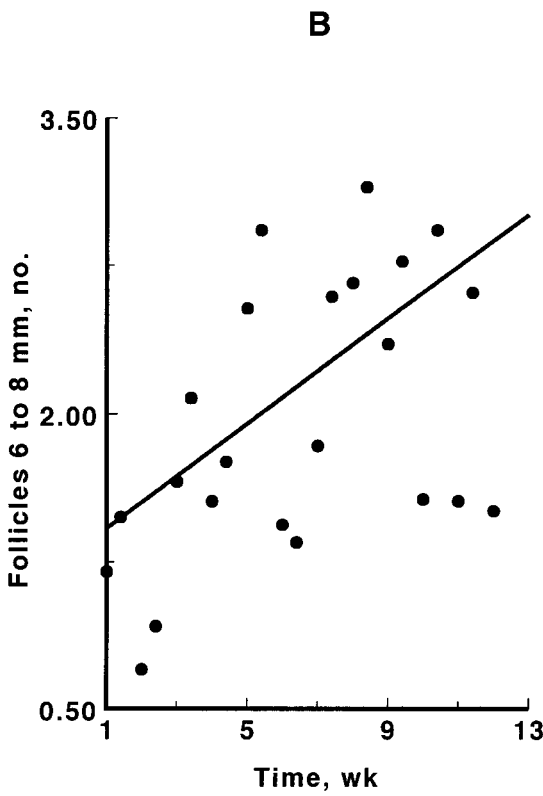
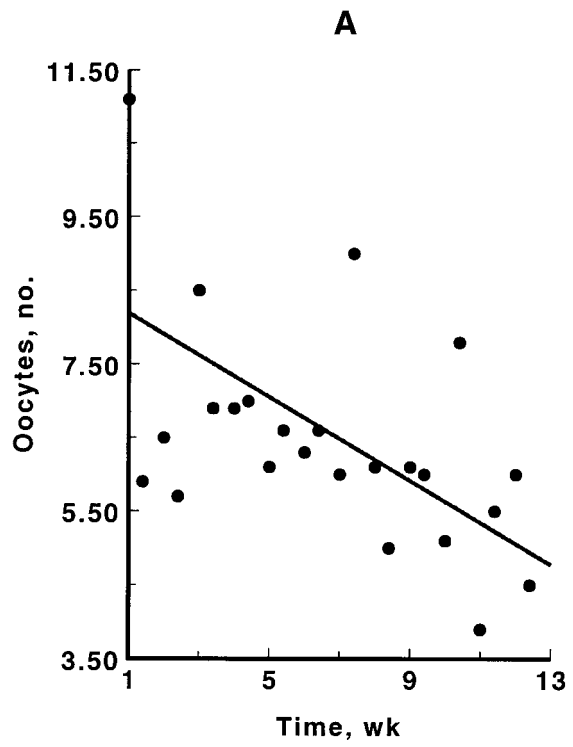
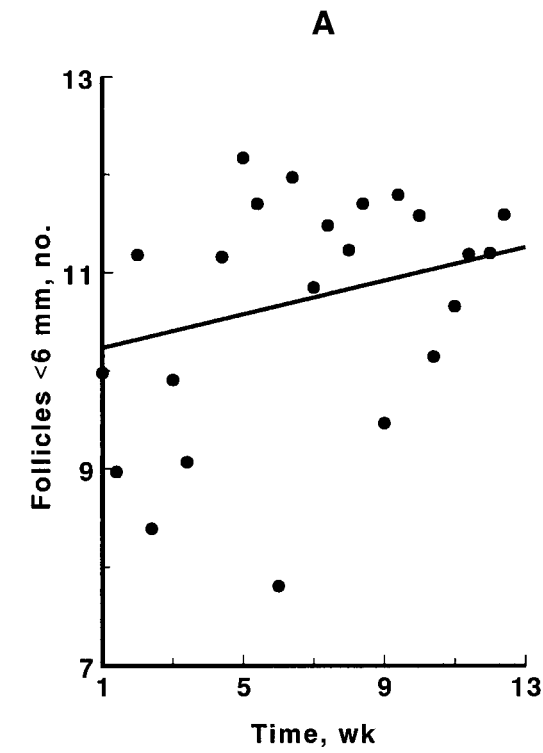


Figure 2. Linear increases in follicles <6 mm (panel A) and 6 to 8 mm in diameter (panel B) through 12 wk of ovum pickup in nonlactating dairy cows with twice weekly aspiration (●, session mean).

Figure 3. Linear decrease in number of oocytes (panel A) and Grade B quality oocytes (panel B) in nonlactating cows aspirated twice weekly for 12 wk (●, session mean).

TABLE 1. The cleavage rate and in vitro development of bovine embryos injected with DNA at 16 h after insemination.

Treatment groups ¹	n	Percentage cleaved			
		Total	n	End stage of development	
				2 to 16 Cells	Morula or blastocyst
1PN injected	178	34.8 ^b	62	54.8	45.2 ^b
1PN control	151	60.9 ^a	92	35.9	64.2 ^{bc}
2PN injected	155	39.4 ^b	61	50.8	49.2 ^{bc}
2PN control	116	71.6 ^a	83	31.3	68.7 ^{ac}
0PN	510	19.0 ^c	97	71.1	28.9 ^{ad}

a,b,c,d Values in same column with different superscripts differ ($P < 0.01$).

¹1PN = Zygotes with 1 pronucleus, 2PN = zygotes with 2 pronuclei, 0PN = oocytes without pronuclei at the time of microinjection.

that developed to morulae or blastocysts was not significantly different ($P > 0.05$) between 1PN injected, 1PN uninjected, and 2PN injected zygotes. However, the development of morulae or blastocysts was highest ($P < 0.01$) in 2PN uninjected than in other zygotes. With presumptive zygotes showing 0PN, the proportion of morula or blastocyst development was significantly lower ($P < 0.01$) than that of all other groups. The proportion of cleaved embryos that arrested at the 2- to 16-cell stage was greatest in the 0PN group (71%) followed by the groups with 1PN injected (54.8%), 2PN injected (50.8%), 1PN control (35.9%), and 2PN control (31.3%).

Effect of Oocyte Quality on Embryo Development

The cleavage rates and subsequent developmental rates of each grade of oocytes are presented in Table 2. Grade A and B oocytes were not different ($P >$

0.05) in cleavage rate or subsequent development to the morula or blastocyst stage. However, their cleavage rates and subsequent development were significantly higher ($P < 0.01$) than were those oocytes of Grade C and D. Grade C oocytes had higher ($P < 0.01$) cleavage and subsequent development rates than did those of Grade D oocytes. The percentage of cleaved embryos that became arrested increased ($P < 0.01$) with decreases in visual quality of oocytes. The percentage of cleaved embryos that arrested at the 2- to 16-cell stage was highest for oocytes of Grade D (73.1%) followed by Grade C (56.3%), B (40.4%), and A (29.3%) when evaluated on d 10 after insemination.

Expression of Green Fluorescent Protein in Embryos

Forty-six percent (148 of 320) of the microinjected embryos expressed integrated DNA by showing green

TABLE 2. Cleavage rate and in vitro development of bovine embryos microinjected with DNA by quality grade of oocytes.

Grade of oocytes ¹	n	Percentage cleaved			
		Total	n	End stage of development	
				2 to 16 Cells	Morula or blastocyst
A	177	55.9 ^a	99	29.3	70.7 ^a
B	207	47.8 ^a	99	40.4	59.6 ^a
C	354	33.6 ^b	119	56.3	43.7 ^b
D	372	21.0 ^c	78	73.1	26.9 ^c

a,b,c Values in same column with different superscripts differ ($P < 0.01$).

¹A = Oocyte with compact multilayered cumulus cells and a homogeneous organized ooplasm, B = oocyte with compact cumulus cells with organized homogenous ooplasm, C = oocyte with less compact cumulus cells and irregular ooplasm containing dark clusters in the ooplasm, and D = oocyte without cumulus cells or with overexpanded cumulus cells and a jelly-like matrix.

TABLE 3. Green fluorescent protein (GFP) expression in bovine in vitro matured-in vitro fertilized embryos.

Oocyte ¹ quality grade	Zygotes injected	GFP- Positive (%)	n	Stage						Development score ²	
				1 cell	2 cells	3 to 8 cells	9 to 16 cells	Morula	Blastocyst	\bar{X}	SE
A	69	53.6	37	35.1	37.8	10.8	0	13.5	2.7	1.3 ^a	0.16
B	83	46.9	39	41.0	43.6	10.3	2.6	2.6	0	0.8 ^b	0.15
C	91	40.6	37	43.2	48.7	8.1	0	0	0	0.6 ^b	0.16
D	77	45.5	35	68.6	31.4	0	0	0	0	0.3 ^c	0.15

^{a,b,c}Values in same column with different superscripts differ ($P < 0.01$).

¹A = oocyte with compact multilayered cumulus cells and a homogeneous organized ooplasm, B = oocyte with compact cumulus cells with organized homogeneous ooplasm, C = oocyte with less compact cumulus cells and irregular ooplasm containing dark clusters in the ooplasm, and D = oocyte without cumulus cells or with overexpanded cumulus cells and a jelly-like matrix.

²Development score was recorded as 1 = 1 to 2 cells, 2 = 3 to 8 cells, 3 = 9 to 16 cells, 4 = morula, and 5 = blastocyst.

cells when evaluated under the fluorescence microscope on d 10 after insemination (Table 3). Grade A oocytes (53.6%) had a greater percentage of GFP positive embryos, followed by Grade B (46.9%), Grade D (45.5%), and Grade C (40.6%). The developmental score of GFP positive embryos was significantly higher ($P < 0.01$) for Grade A oocytes compared with those of Grade B, C, and D. Embryos from Grade B and C oocytes were not different ($P > 0.05$) in their development score. However, embryos from Grade D oocytes had a significantly lower ($P < 0.01$) developmental score than did the Grade A, B, and C oocytes. Among the grades, the maximum numbers of GFP positive embryos were recovered at 1-cell (not cleaved) and 2-cell stages. None of the GFP positive embryos from Grade C and D surpassed the 3- to 8-cell stage of development. Mosaic expression was observed at developmental stages beyond the 3-cell stage (1 embryo at the 4-cell stage had 1 expressing blastomere; 1 embryo at the 8-cell stage had 4 expressing blastomeres; and 1 embryo at the morula stage had half of all blastomeres expressing GFP) by showing some GFP-positive cells.

DISCUSSION

In the present study, a linear increase in the number of follicles was observed throughout the experimental period (12 wk). However, this linear increase in the number of observed follicles did not increase the number of recovered oocytes. This result could be due to the increase in the number of small (<6 mm) follicles, which are difficult to aspirate (22). The recovery rate of oocytes (6.5 ± 3.9 oocytes per cow per aspiration) was low compared with previous reports from our laboratory (14, 15). However, we observed high rates of oocyte recovery in the Grade D category, more than in other grades. In contrast, Santl et al. (25) reported higher recovery

rates in grade A and D oocytes compared with the grade B and C oocytes. The reason for low oocyte recovery is unclear, but it could be related to cow differences (25) or to the technical skills of the individual performing the aspiration (27).

Production of transgenic animals from the oocytes recovered through ovum pickup has a great advantage over the use of slaughterhouse ovaries for two reasons: 1) the genetic potential of animals is known, and 2) disease, which may be transmitted via gametes, can be avoided by using animals known to be disease free. In vitro maturation and IVF techniques have been used successfully to produce transgenic cattle (13, 19). However, to optimize production of transgenic cattle through IVM-IVF, a large number of oocytes is required at the zygote stage for microinjection of DNA, considering the low rate of morula and blastocyst production (12). Even though there has been progress in the optimization of DNA microinjection, large numbers of recipient animals are required for embryo transfer (31). One way to circumvent this problem is to morphologically select only viable preimplantation embryos with an integrated transgene using a marker gene.

The time associated with maximum rate of DNA integration using IVF was reported to be 16 h after insemination when compared with 12 and 24 h (33). At 16 h after insemination, 30% of presumptive zygotes were visualized with 1PN and 24% had 2PN (Table 1). The higher percentage of zygotes visualized with a single pronucleus suggests that formation of the second pronucleus might be delayed, possibly because of late maturation, or subsequent delayed fertilization, or both (7). This hypothesis was further confirmed when no significant differences were observed in the cleavage rates between zygotes with 1PN or 2PN. However, Kubisch et al. (21) observed a 67% cleavage rate in the presumptive zygotes in

which pronuclei were not visualized at 19 h after insemination, which is higher than the cleavage rate in our present study (19%) at 16 h.

After a sperm penetrates an oocyte, the sperm and oocyte chromatin decondense and form pronuclei (33). As the sperm head decondenses, protamines are dissociated from the DNA, and histones become associated during pronuclear formation (1). During this time, the sperm DNA is relatively free of proteins, and DNA replication might occur at this time. Generally, DNA replication has been thought to occur after pronuclear formation. Integration of foreign DNA may occur during replication, possibly because the replicating DNA is more susceptible to breakage and transcriptional activity (21). Use of zygotes that show pronuclear development (1PN, 2PN, or both) for injection with foreign DNA may be advantageous to increase integration frequency. Krisher et al. (19) suggested that microinjection of DNA at 11 h after insemination yielded greater integration efficiencies in microinjected cattle embryos. However, a substantial number of embryos can be left without microinjection as the result of delayed pronuclear formation. In the present study, selection 16 h after insemination for microinjection of any visualized pronucleus (1PN or 2PN) seems to be beneficial, as only 46% of oocytes (mostly from the poor quality oocytes) had no visualized pronucleus.

In the present study, a significantly lower cleavage rate was observed for the injected compared with uninjected presumptive zygotes that had 1PN and 2PN. This result is in agreement with early reports for mice (16), pigs (32), and cattle (18, 19). These studies suggest that microinjection may reduce initial cleavage rates by damaging the pronucleus of the zygote (24).

In the present study, we compared the cleavage rate and subsequent development of oocytes of different quality scores that were microinjected with DNA (Table 2). The results indicate that, following IVM-IVF and microinjection, Grades A and B oocytes had significantly higher cleavage and development rates than did Grades C and D. Grades A and B oocytes had homogeneous cytoplasm and were surrounded by cumulus cells believed to be of sufficient quality to support nuclear and cytoplasmic maturation, male pronuclear formation, and subsequent development *in vitro* (7, 9, 36). Perhaps oocytes with better pronuclear formation, cytoplasmic integrity, and developmental competence can overcome the damage caused by injection procedure.

The incidence of the expression of GFP in murine embryos relates well to the incidence of GFP transgenics in mice (17, 29). However, no such report is

available for the production of transgenic cattle. Selection of GFP-positive embryos was easily performed under the fluorescent microscope. The barrier filter used prevents UV light from being transmitted. In the present study, the positive expression of GFP was higher at the 1- to 2-cell stage and was lower at the morula and blastocyst stages, in agreement with Takada et al. (29). We observed very few mosaic embryos. In contrast, Kubisch et al. (21) reported that a majority of bovine embryos was mosaic when injected with an SV40-LacZ construct. In the present study, a few embryos were mosaic possibly because of events related to transcription or translation that can silence expression selectively in some blastomeres and not in others. Some embryos deemed positive for GFP by PCR methods may have epigenic expression. Further research is required to confirm the integration of GFP by using the embryos with green blastomeres for embryo transfer and subsequent production of a transgenic calf expressing GFP.

The overall GFP expression was higher in Grade A oocytes than in other grades (Table 3). About 14% of morulae and 3% of blastocysts from Grade A oocytes expressed GFP. Similar results (11% of morulae and 3% of blastocysts) were reported in the bovine by Kubisch et al. (21) when zygotes were injected with an SV40-LacZ construct. The higher developmental score of GFP-positive embryos from Grade A oocytes suggests that the quality of the oocyte affects DNA integration in cattle embryos. Conversely, in the present study, the number of embryos expressing integration of DNA was lower than the number of putative transgenic morulae and blastocysts that were identified using PCR procedures (2, 3, 20). The low DNA expression in bovine embryos in the present study could reflect true expression more so than overall integration of GFP. Takada et al. (29) reported in mice that some of the embryos that were GFP-negative had DNA integration as determined by PCR. However, it is likely that PCR analysis leads to an overestimation of integration rates because it cannot distinguish between integrated and nonintegrated DNA (4, 6).

In conclusion, the results of the present study demonstrate that it is efficient to microinject DNA into cattle IVM-IVF zygotes after visualizing 1 or 2 pronuclei at 16 h after insemination. These injected embryos can develop to the preimplantation stage. Furthermore, GFP can be expressed in cultured cattle embryos. More confirmatory data concerning integration into fetal stages or calves are needed before a more exact relationship between the frequency of GFP expression and integration can be known.

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REFERENCES

- 1 Bedford, J. M. 1983. Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol. Reprod.* 33:698-704.
- 2 Behboodie, E., G. B. Anderson, S. Horvat, J. F. Medrano, J. D. Murray, and J. D. Rowe. 1993. Microinjection of bovine embryos with a foreign gene and its detection at the blastocyst stage. *J. Dairy Sci.* 76:3393-3399.
- 3 Bowen, R. A., M. L. Reed, A. Schnieke, G. E. Seidel, A. Stacey, W. K. Thomas, and O. Kajikawa. 1994. Transgenic cattle resulting from biopsied embryos: expression of c-ski in a transgenic calf. *Biol. Reprod.* 50:664-668.
- 4 Burdon, T. G., and R. J. Wall. 1992. The fate of microinjected genes in preimplantation mouse embryos. *Mol. Reprod. Dev.* 33:436-442.
- 5 Brackett, B. G., and G. Oliphant. 1975. Capacitation of rabbit spermatozoa in vitro. *Biol. Reprod.* 12:260-274.
- 6 Canseco, R. S., A.E.T. Sparks, R. L. Page, C. G. Russell, J. L. Johnson, W. H. Velandier, R. E. Pearson, W. N. Drohan, and F. C. Gwazdauskas. 1994. Gene transfer efficiency during gestation and the influence of co-transfer of non-manipulated embryos on production of transgenic mice. *Transgenic Res.* 3:20-25.
- 7 Chian, R. C., K. Okuda, and K. Niwa. 1995. Influence of cumulus cells on in vitro fertilization of bovine oocytes. *Anim. Reprod. Sci.* 38:37-48.
- 8 Chiochetti, A., E. Tolosan, E. Hirsch, L. Silengo, and F. Altruda. 1997. Green fluorescent protein as a reporter of expression in transgenic mice. *Biochim. Biophys. Acta* 1352:193-202.
- 9 Cox, J. F., J. Hormazabal, and M. A. Santa. 1993. Effect of cumulus on in vitro fertilization of bovine matured oocytes. *Theriogenology* 40:1259-1267.
- 10 Cubitt, A. B., R. Heim, S. R. Adams, A. E. Boyd, L. A. Gross, and R. Y. Tsien. 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* 20:448-455.
- 11 De Loos, F., C. van Vliet, P. van Maurik, and A.M.T. Kruip. 1989. Morphology of immature bovine oocytes. *Gamete Res.* 24:197-204.
- 12 Eyestone, W. H. 1994. Challenges and progress in the production of transgenic cattle. *Reprod. Fertil. Dev.* 6:647-652.
- 13 Eyestone, W. H., M. Gowallis, J. Monohan, T. Sink, S. F. Ball, and J. D. Cooper. 1998. Production of transgenic cattle expressing human alpha-lactalbumin in milk. *Theriogenology* 49:386.(Abstr.)
- 14 Gibbons, J. R. W. E. Beal, R. L. Krisher, E. G. Faber, R. E. Pearson, and F. C. Gwazdauskas. 1994. Effects of once versus twice-weekly transvaginal aspiration on oocyte recovery and embryo development. *Theriogenology* 42:405-419.
- 15 Gibbons, J. R., R. L. Krisher, S. K. Carlin, R. E. Pearson, and F. C. Gwazdauskas. 1995. In vitro embryo production after microinjection and ovarian dynamics following transvaginal follicular oocyte aspiration. *Theriogenology* 43:1129-1139.
- 16 Gordon, J. W., G. A. Scangos, D. J. Plotkin, J. A. Barbosa, and F. H. Ruddle. 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. USA* 77:7380-7384.
- 17 Ikawa, M., K. Kominami, Y. Yoshimura, K. Tanaka, Y. Nishimune, and M. Okabe. 1995. A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). *FEBS Lett.* 375:125-128.
- 18 Krimpenfort, P. A., A. Rademakers, W. Eyestone, A. van der Schans, S. van den Broek, P. Kooiman, E. Kootwijk, G. Platenburg, F. Pieper, R. Strijker, and H. de Boer. 1991. Generation of transgenic dairy cattle using in vitro embryo production. *Bio/Technology* 9:844-847.
- 19 Krisher, R. L., J. R. Gibbons, R. S. Canseco, J. L. Johnson, C. G. Russell, D. R. Notter, W. H. Velandier, and F. C. Gwazdauskas. 1994. Influence of the time of gene microinjection on development and DNA detection frequency in bovine embryos. *Transgenic Res.* 3:226-231.
- 20 Krisher, R. L., J. R. Gibbons, F. C. Gwazdauskas, and W. H. Eyestone. 1995. Frequency of detection of exogenous DNA 7, 14, and 21 days after microinjection of bovine zygotes. *Anim. Biotech.* 6:15-25.
- 21 Kubisch, H. M., J. J. Hernandez-Ledezma, M. A. Larson, J. D. Sikes, and R. M. Roberts. 1995. Expression of two transgenes in in vitro matured and fertilized bovine zygotes after DNA microinjection. *J. Reprod. Fertil.* 104:133-139.
- 22 Menard, D. P., P. Lamothe, and L. C. Smith. 1995. Aspiration transvaginale d'ovocytes chez les bovins: mise au point et evaluation d'une technique. *Med. Vet. Quebec* 25:92-96.
- 23 Ninomiya, T., M. Hoshi, A. Masaynagao, and A. Yuki. 1989. Selection of mouse preimplantation embryos carrying exogenous DNA by polymerase chain reaction. *Mol. Reprod. Dev.* 1:242-248.
- 24 Peura, T. T., and J. Janne. 1994. Effect of fluid injection or membrane piercing on the development of in vitro-produced bovine zygotes. *Theriogenology* 41:273.(Abstr.)
- 25 Santl, B., H. Wenigerkind, W. Scherthaner, J. Modl, M. Stojkovic, K. Prella, W. Holtz, G. Brem, and E. Wolf. 1998. Comparison of ultrasound-guided vs. laparoscopic transvaginal ovum pick-up (OPU) in Simmental heifers. *Theriogenology* 50:89-100.
- 26 SAS® User's Guide: Statistics, Version 5 Edition. 1985. SAS Inst., Inc., Cary, NC.
- 27 Scott, C. A., L. Robertson, R. T. de Moura, C. Paterson, and J. S. Boyd. 1994. Technical aspects of transvaginal ultrasound-guided follicular aspiration in cows. *Vet. Rec.* 134:440-443.
- 28 Sirard, M. A., and P. Blondin. 1996. Oocyte maturation and IVF in cattle. *Anim. Reprod. Sci.* 42:417-426.
- 29 Takada, T., K. Lida, T. Awaji, K. Itoh, R. Takahashi, A. Shibui, K. Yoshida, S. Sugano, and G. Tsujimoto. 1997. Selective production of transgenic mice using green fluorescent protein as a marker. *Nat. Biotech.* 15:458-461.
- 30 Wall, R. J. 1997. A new lease on life for transgenic livestock. *Nat. Biotech.* 15:416.
- 31 Wall, R. J., D. E. Kerr, and K. R. Bondioli. 1997. Transgenic dairy cattle: genetic engineering on a large scale. *J. Dairy Sci.* 80:2213-2224.
- 32 Williams, B. L., A.E.T. Sparks, R. S. Canseco, J. W. Knight, J. L. Johnson, W. H. Velandier, R. L. Page, W. N. Drohan, and F. C. Gwazdauskas. 1992. In vitro development of zygotes from prepubertal gilts after microinjection of DNA. *J. Anim. Sci.* 70:2207-2211.
- 33 Xu, K. P., and T. Greve. 1988. A detailed analysis of early events during in vitro fertilization of bovine follicular oocytes. *J. Reprod. Fertil.* 82:127-134.
- 34 Yang, X., C. Kubota, H. Suzuki, M. Taneja, P.E.J. Bols, and G. A. Presicce. 1998. Control of oocyte maturation in cows—biological factors. *Theriogenology* 49:471-482.
- 35 Yoshioka, K., A. M. Othman, T. Taniguchi, H. Yamanaka, and K. Sekikawa. 1997. Differential patterns of blastulation in bovine morulae cultured in synthetic oviduct fluid medium containing FCS or BSA. *Theriogenology* 48:997-1006.
- 36 Zhang, L., S. Jiang, P. J. Wozniak, X. Yang, and R. A. Godke. 1995. Cumulus cells function during bovine oocyte maturation, fertilization and embryo development in vitro. *Mol. Reprod. Dev.* 40:338-344.