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Influence of the DNA amount per microinjection on the development and EGFP expression in bovine embryos

Summary

Development and fluorescence expression were evaluated in bovine embryos microinjected with ~2 pl of DNA-buffer solution containing [I] 1.6, [II] 4.8 or [III] 8.0 ng/µl of the enhanced green fluorescent protein (EGFP) gene, respectively. The percentages of embryo cleavage and development to blastocysts for the DNA-injected groups (Groups I, II and III) were significantly ($p<0.05$) lower than those in the centrifuged control group. However, no significant difference was observed among the DNA-injected and buffer-injected control groups with respect to the percentage of development to blastocysts. There was no significant difference among the DNA injected groups with respect to the percentage of total fluorescing embryos. However, the percentage of total embryos that fluoresced in all the cells (nonmosaic) was significantly ($p<0.01$) higher in Groups II and III than in Group I. These results using the EGFP maker indicate that nonmosaic expression of fluorescence in the microinjected embryos increased by increasing the DNA amount injected. However, those injected embryos exhibited impaired development irrespective of the DNA dose.

Key Words: bovine, embryos, fluorescence, microinjection, EGFP

Zusammenfassung

Titel der Arbeit: **Zum Einfluss der mikroinjizierten DNA-Menge auf die Entwicklung und EGFP-Expression in Rinderembryonen**

In Embryonen des Rindes wurden ca. 2 pl DNA-Lösung mit [I] 1,6, [II] 4,8 oder [III] 8,0 ng/µl des Gens für das grün fluoreszierende Protein (EGFP) injiziert. Nachfolgend wurde die Fluoreszenz sowie die Entwicklung der Embryonen untersucht. Die Teilungsrate und die Blastozystenrate waren in den Gruppen I, II und III signifikant ($P<0,05$) geringer als in der Kontrollgruppe (zentrifugierte Embryonen ohne Injektion). Die Entwicklung von Embryonen zu Blastozysten war nach Injektion DNA-haltiger und DNA-freier Lösung nicht signifikant verschieden. Der Anteil fluoreszierender Embryonen unterschied sich nicht signifikant zwischen den Gruppen I bis III. In den Gruppen II und III war jedoch der Anteil Embryonen, bei denen alle Zellen fluoreszierten, signifikant höher ($P<0,01$) als in der Gruppe I. Die Ergebnisse mit dem EGFP-Marker zeigen, dass steigende DNA-Mengen je Mikroinjektion mit einer Zunahme des Anteils Embryonen verbunden sind, bei denen alle Zellen Fluoreszenzsignale senden. Die Entwicklungskompetenz der Embryonen nimmt nach Mikroinjektion, unabhängig von der injizierten DNA-Menge, ab.

Schlüsselwörter: Rind, Embryonen, Fluoreszenz, Mikroinjektion, EGFP

Introduction

Transgenic dairy animals have been developed in various species for use as bioreactors producing pharmaceutical products (STICE et al., 1998; ZIOMEC, 1998; CHAN, 1999). Low efficiency of producing transgenic livestock by microinjection, however, has been a major problem, especially in large animals, such as cattle. One way of circumventing the difficulty would be the application of green fluorescent protein (GFP) as a marker for the selection of transgenic embryos prior to their transfer to

recipient females. This approach has been under investigation to verify the simplicity and reliability of the GFP marker for the transgenic embryo selection in mice and cattle (TAKADA et al., 1997; CHAN et al., 1999; CHAUHAN et al., 1999; KATO et al., 1999; MURAKAMI et al., 1999). However, low frequency of fluorescence expression and a high rate of the mosaic expression have been demonstrated particularly in bovine embryos after microinjection of the various mutant GFP genes. Some of the important parameters that could influence successful integration of foreign DNA into mouse chromosome, such as DNA concentration, size and form, injection buffer and the site of injection, have been described previously (BRINSTER et al., 1985). However, the optimal conditions for the DNA insertion are poorly understood in cattle.

The objective of this study was to examine the influence of increasing the number of DNA copies including an enhanced GFP (EGFP) marker gene (OKABE et al., 1997) per microinjection on the developmental potential and fluorescence expression in bovine embryos.

Materials and Methods

In vitro fertilization

The methods used for *in vitro* maturation, *in vitro* fertilization (IVF) and subsequent culture in the experiments were modified from the procedure previously described by OTOI et al. (1993). Briefly, ovaries were collected from cows at a local abattoir, and cumulus-oocyte-complexes (COC) were aspirated from the follicles (2 - 7 mm in diameter). After aspiration, the COC were cultured in 25 mM HEPES TCM-199 with Eagle's salts (Gibco, Grand Inland, NY) supplemented with 5 % fetal bovine serum (FBS; Sebak, Aidenback, Germany), 0.01 mg/ml follicle stimulating hormone (Denka, Kawasaki, Japan) and 50 μg/ml gentamicin (Sigma, St. Louis, MO) at 38^{o}C under 5 % CO_2 in air. After 20 to 22 h of culture, the COC were fertilized *in vitro* with frozen-thawed sperm for 5 h in Brackett and Oliphant medium (BRACKETT and OLIPHANT, 1975) containing 2.5 mM caffeine (Sigma), 3 mg/ml bovine serum albumin (BSA; Sigma), 20 μg/ml heparin (Shimizu Pharmaceuticals, Shimizu, Japan) and then cultured *in vitro* in TCM-199 supplemented with 5 % FBS, 5 μg/ml insulin (Sigma) and 50 μg/ml gentamicin.

Pronuclear microinjection

At 17 h after IVF, the cumulus cells of these sperm-exposed oocytes were discarded by vortexing in Dulbecco's phosphate-buffered saline (DPBS; Gibco) containing 0.1 % hyaluronidase (Sigma) for 4 min (day of IVF = day 0). The denuded oocytes were then centrifuged at 16,000 x g for 10 min in DPBS containing 3 mg/ml BSA, 5 μg/ml cytochalasin B and 50 μg/ml gentamicin (manipulation solution) to visualize the male and female pronuclei. The EGFP cDNA fragment under control of the chicken beta-actin promoter and cytomegalovirus enhancer (OKABE et al., 1997) was diluted with the buffer solution (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.5) to the final concentrations of [1] 1.6, [2] 4.8 and [3] 8.0 ng/μl, respectively. The DNA-buffer solution of each concentration was microinjected into the pronuclei of the sperm-exposed oocytes (~2 pl) in a 200-μl droplet of the manipulation solution. These

injected oocytes were cultured on a cumulus cell mono-layer for an additional 7 days (day 8) to evaluate their developmental competence and fluorescence expression.

Embryo development and fluorescence

The developmental stage and fluorescence expression in these microinjected embryos have been monitored using a fluorescence stereomicroscope (MZ-12 with GFP Plus filter, Leica) until day 8. The embryos showing fluorescence in all their blastomeres were classified as having "whole expression". The fluorescent embryos containing nonfluorescent blastomeres in the group were termed as having mosaic expression.

Statistic analysis

Cleavage and blastocysts rates were compared among the injected and centrifuged control embryos, and the rates of fluorescence expression were compared among the embryos injected with different concentrations of the DNA using χ^2 analysis and the Fisher exact probability test. Differences at a probability value of 0.05 or less were considered significant in this study.

Results

The developmental property of the embryos microinjected with various concentrations of the EGFP gene is shown in Table 1. Statistic comparisons were not made between the nontreated control and the other groups because the fertilized oocytes in control group could not be selected for the presence of pronuclei. Oocytes observed having 2n pronuclei were selected in centrifuged control group. The percentages of cleaved embryos and development to blastocysts for the DNA-injected groups (Groups I, II and III) were significantly lower ($p<0.05$) than for the centrifuged control group. In addition, the percentage of cleaved embryos for Groups II and III was significantly lower ($p<0.05$) than for lowest concentration Group I and buffer-injected control group. However, there was no significant difference among the DNA-injected and buffer-injected control groups with respect to the percentage of development to blastocysts.

Table 1
Development in vitro of bovine embryos microinjected with different concentrations of the EGFP gene

Concentration of DNA (ng/µl)	No. examined (n)	No. (%) embryos	
		Cleaved	Developed to blastocysts
[I] 1.6	330	132 (40.0)[d]	19 (5.8)[d]
[II] 4.8	50	10 (20.0)[e]	4 (8.0)[d]
[III] 8.0	96	27 (28.1)[e]	4 (4.2)[d]
Buffer-injected[a]	103	45 (43.7)[df]	6 (5.8)[d]
Centrifuged[b]	48	28 (58.3)[f]	11 (22.9)[e]
Control[c]	84	60 (71.4)	19 (22.6)

[a] Control oocytes microinjected with the buffer only after centrifugation.
[b] Control oocytes observed having 2n pronuclei after centrifugation.
[c] Nontreated sperm-exposed oocytes that had not been selected for the presence of pronuclei.
[d-f] Values in a column with the different letters are significantly different ($p<0.05$).

Pattern and frequency of fluorescence expression by the embryos injected with different concentrations of the DNA were summarized in Table 2. There were no significant differences among the DNA-injected groups with respect to the percentage of the total fluorescing (whole and mosaic) embryos. However, percentage of the total

embryos that fluoresced with whole expression was significantly greater ($p<0.01$) in higher concentration Groups II and III than in Group I.

Table 2
Pattern and frequency of fluorescence expression by bovine embryos microinjected with different concentrations of the EGFP gene

	[I]	[II]	[III]
DNA (ng/μl)	1.6	4.8	8.0
n	330	50	96
No. (%) fluorescing 1-cell embryos	8 (2.4)	3 (6.0)	4 (4.2)
No. (%) fluorescing embryos with			
Mosaic expression			
2 to 16 cells	33 (10.0)	3 (6.0)	12 (12.5)
Morula	4 (1.2)	0 (0.0)	0 (0.0)
Blastocyst	3 (0.9)	0 (0.0)	0 (0.0)
Total	40 (12.1)	3 (6.0)	12 (12.5)
Whole expression			
2 to 16 cells	8 (2.4)[a]	4 (8.0)[ab]	8 (8.3)[b]
Morula	0 (0.0)	0 (0.0)	0 (0.0)
Blastocyst	1 (0.3)	1 (2.0)	1 (1.0)
Total	9 (2.7)[a]	5 (10.0)[b]	9 (9.4)[b]
Total no. (%) fluorescing embryos	57 (17.3)	11 (22.0)	25 (26.0)

[a-b] Values in a column with the different letters are significantly different ($p<0.05$).

Discussion

The pronuclei of bovine sperm-exposed oocytes were microinjected with 1-, 3- or 5-fold higher concentrations of the DNA solution than the standard level (1.6 ng/μl, containing 1,000 copies of the EGFP gene per 2 pl) used in mice (OKABE et al., 1997). BRINSTER et al. (1985) demonstrated that injection of large amounts of DNA in mice eggs was toxic. In this study, the oocytes injected with high concentration of DNA (3- and 5-folds) had lower cleavage rates, as compared with those injected with a standard DNA concentration (1-fold). This difference, however, was not mirrored by the number of blastocysts that developed in each injected group. Furthermore, even in the buffer-injected control group, the percentage of embryos developed to blastocysts decreased significantly as compared with the centrifuged control group. These results imply that the developmental property of the embryos was less affected by the concentration of DNA injected than the experimental process itself, such as pronuclear damage by microinjection that may reduce initial cleavage rates of zygotes (CHAUHAN et al., 1999). Moreover, it has also been demonstrated that pronuclear microinjections with either water or buffer decreased bovine embryonic development significantly (PEURA et al., 1995). Therefore, the reduced percentage of development to blastocysts in the injected groups might be due, in part, to the microinjection with buffer itself regardless of the DNA amount included.

There was no significant difference among the embryos injected with different concentrations of the DNA with respect to the percentages of the total fluorescing embryos. However, the percentage of the total embryos expressing fluorescence in all

their cells (nonmosaic) increased significantly by increasing the DNA concentration injected per oocyte. Also, percentage of the total embryos with nonmosaic expression was significantly higher ($p<0.01$) in the higher DNA concentration groups (3- and 5-folds) than the standard DNA concentration group (1-fold), when the rates were based on the total of cleaving embryos [3-fold; 50.0 % (5/10), 5-fold; 33.3 % (9/27) vs. 1-fold; 6.8 % (9/132)]. Although the treatment numbers were restricted in the present study, higher proportions of the injected embryos tended to develop to blastocysts with uniform marker expression in higher DNA concentration groups (Table 2). Integration of the microinjected DNA into the genome of the recipients may or may not have occurred, however, delayed gene insertion has been suggested as one cause of transgenic mosaicism (CHAN et al., 1999). It has also been demonstrated, in mice study, that integration efficiency improved as the DNA concentration in the injection fluid increased (BRINSTER et al., 1985). Furthermore, in bovine embryos with 100 % blastomeres expressing GFP, the integration event was predicted to have occurred during the first cell cycle at the pronuclear stage before DNA replication (CHAN et al., 1999). The results obtained in the present study, therefore, suggest that the insertion frequency at the earlier pronuclear stage might be improved by increasing the amount of the DNA copies microinjected.

In conclusion, results using the EGFP marker gene suggest that higher numbers of the embryos with nonmosaicism were generated by increasing the concentration of the DNA in the buffer solution microinjected. However, the potential problems of impaired development and low efficiency of transgene expression could not be solved. Further studies, such as nuclear transfer and transsomatics, will be required to improve the efficiency of producing transgenic bovine embryos.

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References

BRACKETT, B.G.; OLIPHANT, G.:
Capacitation of rabbit spermatozoa in vitro. Biol. Reprod. **12** (1975), 260-274

BRINSTER, R.L.; CHEN, H.Y.; TRUMBAUER, M.E.; YAGLE, M.K.; PALMITER, R.D.:
Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc. Natl. Acad. Sci. U. S. A. **82** (1985), 4438-4442

CHAN, A.W.S.; KUKOLJ, G.; SKALKA, A.M.; BREMEL, R.D.:
Timing of DNA integration, transgenic mosaicism, and pronuclear microinjection. Mol. Reprod. Dev. **52** (1999), 406-413

CHAN, A.W.S.:
Transgenic animals: Current and alternative strategies. Cloning **1** (1999), 25-46

CHAUHAN, M.S.; NADIR, S.; BAILEY, T.L.; PRYOR, A.W.; BUTLER, S.P.; NOTTER, D.R.; VELANDER, W.H.; GWAZDAUSKAS, F.C.:
Bovine follicular dynamics, oocytes recovery, and development of oocytes microinjected with a green fluorescent protein construct. J. Dairy Sci. **82** (1999), 918-926

KATO, M.; YAMANOUCHI, K.; IKAWA, M.; OKABE, M.; NAITO, K.; TOJO, H.:
Efficient selection of transgenic mouse embryos using EGFP as a marker gene. Mol. Reprod. Dev. **54** (1999), 43-48

MURAKAMI, M.; FAHRUDIN, M.; VARISANGA, M.D.; AND SUZUKI, T.:

Fluorescence expression by bovine embryos after pronuclear microinjection with the EGFP gene. J. Vet. Med. Sci. **61** (1999), 843-847

OKABE, M.; IKAWA, M.; KOMINAMI, K.; NAKANISHI, T.; NISHIMUNE, Y.:
'Green mice' as a source of ubiquitous green cells. FEBS Lett. **407** (1997), 313-319

OTOI, T.; TACHIKAWA, S.; KONDO, S.; SUZUKI, T.:
Developmental capacity of bovine oocytes frozen in different cryoprotectants. Theriogenology **40** (1993), 801-807

PEURA, T.T.; TOLVANEN, M.; HYTINEN, J-M.; JANNE, J.:
Effects of membrane piercing and the type of pronuclear injection fluid on development of in vitro-produced bovine embryos. Theriogenology **43** (1995), 1087-1096

STICE, S.L.; ROBL, J.M.; PONCE De LEON, F.A.; JERRY, J.; GOLUEKE, P.G.; CIBELLI, J.B.; KANE, J.J.:
Cloning: New breakthroughs leading to commercial opportunities. Theriogenology **49** (1998), 129-138

TAKADA, T.; IIDA, K.; AWAJI, T.; ITOH, K.; TAKAHASHI, R.; SHIBUI, A.; YOSHIDA, K.; SUGANO, S.; TSUJIMOTO, G.:
Selective production of transgenic mice using green fluorescent protein as a marker. Nature Biotechnol. **15** (1997), 458-461

ZIOMEK, C.A.:
Commercialization of proteins produced in the mammary grand. Theriogenology **49** (1998), 139-144

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