0.035, respectively). In addition, at 4 weeks of age, the *in vivo* males became significantly lighter when compared to the naturally mated males ($P = 0.034$). At 8 weeks of age, the *in vivo* females had a significantly elevated systolic blood pressure when compared to the *in vitro* females ($P = 0.003$); however, at 21 weeks of age, both *in vitro* males and females had a significantly elevated blood pressure when compared to *in vivo* offspring ($P < 0.003$). At 8, 15, and 21 weeks of age, offspring derived from transferred embryos developed with significantly elevated systolic blood pressure when compared to non-embryo transfer offspring ($P < 0.05$). No significant differences in serum angiotensin-converting enzyme activity (a potent regulator of systolic blood pressure) was observed between the treatment groups. Significantly altered liver:body weight ratios were observed between the *in vitro* and *in vivo* males, and between the *in vitro* and the naturally mated (6) females ($P < 0.038$). All of the above data are independent of litter size. These data support the hypothesis that early embryo environment can influence postnatal growth and cardiovascular physiology.

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151 EFFECTS OF HEXOSES SUPPLEMENTED IN THE MATURATION AND PRE IMPLANTATION MEDIUM ON THE *IN VITRO* DEVELOPMENT OF PORCINE OOCYTES

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The present study was conducted to determine the effects of hexoses on *in vitro* maturation, fertilization, and development of porcine oocytes. In the first experiment, porcine oocytes were matured in modified North Carolina State University (NCSU)-37 medium supplemented with 0.6 mM cysteine, 1 mM dibutyryl cyclic AMP (dbc AMP), 10 IU/mL equine chorionic gonadotrophin, 10 IU/mL human chorionic gonadotrophin, 50 μ/mL gentamycin (Sigma-Aldrich, Tokyo, Japan), 10% (v/v) porcine follicular fluid, and various hexoses (glucose, fructose, and galactose) at various concentrations of 0 (control), 2.5, 5.5, and 10 mM. They were subsequently cultured in the maturation medium without hormones and dbcAMP for an additional 22 h. Fertilization was performed according to Kikuchi *et al*. (2002 Biol. Reprod. 66, 1033–1041); 15 oocytes were co-incubated with 1 million frozen thawed sperm/mL in fertilization medium for 5 h. Supplementation of either glucose (2.5 or 5.5 mM) or fructose (5.5 mM) in the maturation medium significantly increased the percentages of maturation to metaphase II (68.5%, 79.4%, and 70.2%, respectively) and monospermic fertilization of oocytes (55.0%, 64.5%, and 58.9%, respectively), as compared with control group (metaphase II: 52.8%; monospermic: 42.7%; $P < 0.05$). Supplementation of galactose had no effect on the meiotic maturation and monospermic fertilization of oocytes. In the second experiment, presumptive zygotes were cultured in modified NCSU-37 supplemented with 4 mg/mL BSA, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, and 50 μg/mL gentamycin. The cleaved embryos were collected at Day 3 after *in vitro* fertilization and then cultured for a further 4 days in modified NCSU-37 medium supplemented with 5.5 mM of glucose, fructose, or galactose. The percentages of blastocyst formation, calculated from cleaved embryos, were significantly higher in the glucose and fructose groups (20.4% and 18.4%, respectively) than in the galactose group (12.9%) and the non-supplemented control group (9.2%). Although fructose supplementation did not accelerate blastocyst formation, it did significantly increase the mean cell number of blastocysts (48.0 ± 2.9 vs. 38.6 ± 1.8) and reduced the index of DNA-fragmented nuclei in the blastocysts stained by the TUNEL method (7.6 ± 0.9 vs. 11.8 ± 0.9), as compared with glucose supplementation. In the present study, although all hexoses were used through the glycolysis pathway, supplementation of galactose in the maturation and embryo culture medium had no beneficial effect on development. In contrast, both glucose and fructose enhanced development with an optimal concentration of 5.5 mM. Replacement of glucose with fructose did not accelerate blastocyst formation but did enhance embryo quality in terms of increasing cell number and decreasing the number of fragmented nuclei.

Embryo Manipulation

152 *IN VITRO* AND *IN VIVO* DEVELOPMENT OF BOVINE IVP EMBRYOS FOLLOWING SINGLE-CELL BIOPSY ON DAY 4

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Maximum advantage can be gained from gene discovery programs, by screening embryos carrying the desired genes(s) prior to immediate transfer. This requires an efficient and reliable genotyping system and a method for biopsy preparation that does not compromise subsequent embryo or fetal development. The present study examined the effect of removing a single-cell from the developing 8–16 cell embryo on its subsequent ability to continue development to at least the late morula stage *in vitro* and then survive following triple transfer to recipients. Abattoir-sourced ovaries were obtained and subjected to IVP as previously described (van Wagtendonk-De Leeuw AM *et al*. 2004 Reprod. Fert. Dev. 16, 214 abst). Briefly, oocytes were matured in TCM199 +10% FCS, 10 μg/mL FSH, 10 μg/mL LH, 1 μg/mL estradiol, and 100 μM cysteamine under 5% CO_2 in air at 38.5°C for 24 h. Percoll-separated sperm (1×10^6/mL) were then co-incubated with the matured oocytes (Day 0) for 24 h with the presumptive zygotes further cultured in mSOF medium under 5%CO_2, 7% O_2, 88% N_2. On Day 4 embryos with a minimum of 8 cells were selected and held at 38.5°C in HEPES-buffered SOF (HSOF) until biopsy at ambient temperature. Embryo biopsy was performed in HSOF medium + 5 μg/mL cytochalasin B. A single cell was removed using a 30 μm biopsy pipette. Both biopsied and control embryos were then further cultured in mSOF in individual

wells prepared in a 1% agarose matrix (Peura TT 2003 Cloning Stem Cells 5, 13–24). Embryos were scored for grade and stage of development reached on Day 7, and Grades 1 and 2 blastocysts and expanded blastocysts were transferred to synchronized recipients (three embryos of the same stage and grade to each recipient; $n = 50$). Fetal number was determined on Day 35 and 62 of gestation. A model for embryo survival was fitted to the data (McMillan WH *et al*. 1998 Theriogenology 50, 1053–1070) in order to estimate embryo ("e") and recipient ("r") contributions to embryo survival. Values were then compared to those determined for control embryos, produced using identical IVP methods (van Wagtendonk-De Leeuw AM *et al*. 2004 Reprod. Fert. Dev. 16, 214 abst). A total of 358 control and 561 biopsied embryos were cultured. Removal of a single cell did not significantly affect *in vitro* development (60.1% vs. 56.0%; control vs. biopsy). Day 35 survival of biopsied embryos was 44.7% with calculated "e" and "r" values of 0.48 and 0.94, respectively, which did not differ from control values (44.1%; 0.50 and 0.89). However, by Day 62 fetal survival had significantly decreased with a concomitant drop in "e" but not "r" (30.0%; 0.32 and 0.94, respectively; control "e" and "r" were unchanged). In conclusion, single-cell biopsy of the 8–16-cell embryo does not affect *in vitro* development or embryo survival to Day 35. However, significant fetal loss occurs by Day 62 that may limit commercial application. Further work is required to elucidate the cause of and overcome fetal loss.

153 EFFECTS ON SEX RATIO AND PREGNANCY RATES OF *IN VIVO*-DERIVED BOVINE EMBRYOS USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION SEXING METHOD

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In this study, we examined the effects of developmental stages and quality grades on sex ratio of *in vivo*-derived bovine embryos. Furthermore, pregnancy rates of fresh frozen-thawed sexed embryos or intact (non-sexed) fresh and frozen-thawed embryos were compared in order to efficiently carry out the sexing of embryos in the field. Embryos were collected from donors at 7 days after estrus following a routine superovulation protocol, and classified into four stages (late morula, early blastocyst, blastocyst, and expanded blastocyst) and two quality grades (Grade 1 and Grade 2–3) by the IETS manual. Embryos were frozen by direct transfer method from 1 to 3 h post-collection in 0.25-mL straws as described previously (Aoyagi *et al.* 1996 Theriogenology 45, 165 abst). Frozen embryos were thawed in 30°C water for 20 s following 7 s in air. They were then squeezed out into PBS + 5% FCS (PBS), washed twice, and incubated in CR1aa + 5% CS (CR1aa) or PBS. Recently, a commercial embryo sexing program was performed at our laboratory using loop-mediated isothermal amplification (LAMP). The procedure takes 5 min to perform each embryo biopsy and only 40 min for the LAMP process. A few cells of fresh (F; $n = 105$) and frozen-thawed (Z; $n = 143$) embryos of Grade 1 (H), and fresh (F; $n = 77$) embryos of Grade 2–3 (L) were biopsied with a microsurgical blade, and sex was determined by the LAMP method. Embryos were transferred non-surgically into heifers on Day 7 of the estrus cycle. Pregnancies were determined by ultrasonography on Day 30. Data were analyzed by the chi-square test. The sexing of all 325 embryos yielded 148 female (46%), and only 2 embryos were indeterminant (1%). There was no evidence of any effect of developmental stage on sex ratio (female embryos: late morula 69/157 (44%), early blastocyst 42/94 (45%), blastocyst 29/53 (55%), and expanded blastocyst 10/21 (48%)). However, when the sex ratio was examined for embryos of different quality grades, significantly more females were found in the embryos appearing more degenerated (female embryos: FH + ZH vs. FL; 42% vs. 57%, $P < 0.05$). Pregnancy rates on Day 30 with FH embryos (38/45, 84%) were similar to rates obtained with non-sexed fresh (60/81, 78%) and frozen-thawed embryos (44/54, 82%). The pregnancy rates on Day 30 with ZH embryos incubated in CR1aa (18/40, 45%) were lower than those of FH, non-sexed fresh, and frozen-thawed embryos. However, pregnancy rates of ZH embryos incubated in PBS (13/16, 81.3%) were significantly higher than for those frozen embryos that were thawed and incubated in CR1aa ($P < 0.05$). After the transfer of embryos sexed by the LAMP method to recipient animals, all 55 calves born were of the predicted sex. In conclusion, the present results showed that with the LAMP method for sexing of the embryos, there were only a few samples for which sex could not be determined. Examination of *in vivo*-derived Day 7 embryos indicated that female embryos graded lower than male embryos. Furthermore, the removal of a few cells from a fresh or frozen-thawed embryo did not impact its subsequent viability.

154 EFFECTS OF BLASTOMERE BIOPSY AND OXYGEN CONCENTRATION DURING CULTURE ON THE DEVELOPMENT AND INTERFERON-TAU SECRETION OF BOVINE EMBRYOS

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Pre-implantation genetic diagnosis has become a powerful tool in human *in vitro* fertilization. Yet, for ethical reasons, controlled experiments assessing the effects of embryo biopsies on human embryos are difficult to perform. Therefore, a series of experiments was performed to examine the effects of blastomere biopsies on subsequent development of IVF-derived bovine embryos cultured under either atmospheric or low (5%) oxygen. Embryos were generated by IVF of *in vitro*-matured oocytes and cultured in CR1aa medium containing 5% bovine fetal serum. The first experiment was designed to assess the optimal time for blastomere removal. One blastomere was removed from each of a large number of embryos at either 48 or 72 h after IVF. Biopsy at 48 h resulted in 17.2% (10/58) of embryos proceeding to the blastocyst stage, which was significantly lower than when biopsies were performed at 72 h (37.5% (33/88), $P < 0.05$). In the second experiment, embryos were cultured under either atmospheric or 5% O_2 following blastomere removal. Biopsies at 72 h had no effect on rate of blastocyst formation with 38.5% (110/286) of controls and 33.7% (60/178) of biopsied embryos proceeding to the blastocyst stage. However, culture under 5% O_2 significantly increased the number of blastocysts from 29.9% (69/231) to 43.3% (101/233). This effect was significant in both biopsied and control embryos. In contrast, staining of blastocysts for cell numbers did not reveal any effects of biopsies or culture conditions. In the final experiment, biopsies were again performed at 72 h and embryos were cultured as previously. Blastocysts were collected and cultured individually for 48 h in 50-μL-medium droplets in their respective O_2 concentrations after which time the medium was assayed for concentration of interferon-tau (IFN-τ). Previous work has shown that IFN-τ secretion can be affected by genetic and environmental factors. Reduced O_2 concentration again significantly increased blastocyst formation from

24.9 to 36.9% (49/197 vs. 75/203, $P < 0.05$), while blastomere removal had no effect on development. IFN-τ secretion did not differ between biopsied and control blastocysts, although culture under atmospheric O_2 resulted in significantly increased IFN-τ concentration in medium droplets (12,690.1 ± 2715.6 vs. 4726.8 ± 2488.5 pM, $P < 0.05$).

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155 DEVELOPMENT OF BOVINE AGGREGATE EMBRYOS CONSTRUCTED FROM NUCLEAR TRANSFER EMBRYOS AND ELECTROFUSED IVF-DERIVED EMBRYOS

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The production of animals by nuclear transfer can be hindered by placental and developmental abnormalities in the fetus. Studies in mice have indicated that tetraploid embryo complementation can be used to rescue embryos with lethal placental deficiencies and produce live offspring. The objectives of this experiment were to produce bovine electrofused embryos by blastomere fusion and to utilize those embryos for aggregation with nuclear transfer (NT) embryos. Oocytes were obtained from a commercial source (BoMed, Madison, WI, USA) and were allocated for use in either NT or *in vitro* fertilization (IVF). NT embryos were produced using standard procedures. Bovine transgenic fibroblast cells maintained in active culture were used as the nuclear donor. Oocytes were fertilized with frozen semen using standard IVF procedures. Zygotes were observed for cleavage and selected at the 2-cell stage for electrofusion at 28, 30, 32, and 34 h post-insemination in 0.3 M mannitol fusion buffer. Embryos were aligned using a 5 s, 7.5 V AC pulse and were fused using a 1.4 kV cm^{-1} 100 μs DC pulse. Treated embryos were observed after ~1 h for fusion of cell membranes between the two blastomeres and were returned to culture (IVF-Fused). Good quality 8-cell embryos produced from the NT and IVF-Fused groups were selected for aggregation at 72 h post-insemination. Aggregate embryos were constructed by removing 3–4 blastomeres from an 8-cell NT embryo. The zona pellucida of an 8-cell IVF-Fused embryo was then removed by placing the embryo in a 0.25% pronase solution for approximately 1 min. After the zona pellucida was removed, 3–4 blastomeres were aspirated from the IVF-Fused embryo and were injected into the NT embryo using a glass pipette. Embryos were returned to culture in CR1aa media and were examined at 168 and 192 h post-insemination for blastocyst (BLST) development (Table 1). This study demonstrates that NT/IVF-Fused aggregate embryos can be constructed and develop at the same rate as controls. An attempt was made to determine the nuclear status of the electrofused embryos but technique limitations did not permit differentiation between tetraploid and multinucleate cells. Further research is needed to determine nuclear status of IVF-Fused embryos and the allocation of NT and IVF-Fused cell lineages within the developing embryo.

Table 1. Blastocyst rates of NT/IVF-Fused aggregate embryos constructed at the 8-cell stage

Treatment	$n^{\dagger}$	No. BLST 168 h (%)	No. BLST 192 h (%)
IVF-Fused Control	58	30 (52)[a]	35 (60)[a]
NT Control	115	64 (56)[a]	66 (57)[a]
NT/IVF-Fused Aggregates	115	56 (49)[a]	61 (53)[a]

[†] Number of 8-cell embryos.
Calculated from number of 8-cell embryos.
[a] No significant difference between groups was detected with a χ^2 analysis ($P < 0.05$).

156 ENHANCED BOVINE EMBRYONIC DEVELOPMENT AFTER MICROFLUIDIC CUMULUS CELL REMOVAL POST-FERTILIZATION

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Microfluidic technologies are increasingly being used in cell biology and embryology research. In order to manipulate an embryonic environment microfluidics take advantage of miniscule media amounts. With the use of pressure heads and laminar flow profiles, a presumptive zygote can be gently manipulated in a microfluidic device for removal of the supporting cumulus cells post-fertilization. Presumptive embryos were assigned at random to three cumulus removal treatments at 48 h post-fertilization: vortexing (3 min), handstripping (with 135-μm-ID stripping pipette), and microfluidics. Blastocyst rates were determined through Day 8 post-fertilization. Rates were analyzed by the GENMOD procedure in SAS (SAS Institute, Inc., Cary, NC, USA), accounting for replicates and treatment. Kinetics of development were also impacted as larger proportions of embryos in the microfluidic group reached the blastocyst stage before embryos of the vortex or hand-stripping treatments. These data suggest that cumulus cell removal in a gentle fashion is associated with enhanced embryonic development in the bovine.

Table 1. Comparison of cumulus cell removal techniques on bovine blastocyst rates

Treatment	Total embryos	Day 8 blastocysts	% Development
Vortexing	521	44[a]	8.4
Handstripping	644	99[b]	15.4
Microfluidics	529	135[c]	25.5

[a–c] Values with different symbols are significantly different; $P = 0.0004$.

157 HOLSTEIN-CHINESE YELLOW HYBRID RECIPIENT OOCYTES RECOVERED BY OVUM PICKUP CAN IMPROVE THE DEVELOPMENT OF CLONED BOVINE EMBRYOS

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We used the subspecies hybrid F1 oocytes (Holstein/Chinese Yellow cattle) recovered by ovum pickup (OPU) as recipient cytoplasts to improve the development of bovine cloned embryos. Ten Holstein cattle, four Chinese Yellow cattle, and four hybrid F1 bovines were subjected to OPU once a week. In total, 44, 110, and 42 OPU sessions were respectively performed for hybrid cattle, Holstein cattle and Chinese Yellow cattle. The mean numbers of punctured follicles for hybrid and Yellow cattle were higher than for Holstein cattle (11.4 ± 0.5 and 11.7 ± 0.5 vs. 10.1 ± 0.7, mean $\pm$ SE), but the recovery rate for Holstein cattle was higher than that for hybrid and Yellow cattle (76.2% vs. 70.3% and 66.6%); therefore, recovered oocytes per session were similar in hybrid, Holstein, and Yellow cattle (8.0 ± 0.5, 7.7 ± 0.4 and 7.8 ± 0.5, respectively). No difference was shown in the quality of the recovered oocytes among the three breeds. The three kinds of recipient oocytes had a maturation rate of 72–73% (256/353, 614/847, and 238/327, respectively). Matured oocytes were used as recipients without selection, and same batch cumulus cells collected from Holstein cow were used as donor cells. The nuclear transfer procedure was essentially as described by Park *et al*. (2004 Mol. Reprod. Dev. 69, 365–374). Cleavage rate of reconstructed embryos was similar in the hybrid, Holstein, and Yellow groups (66%, 66%, and 75%, respectively). However, the blastocyst rate from the cleavage embryos (51% vs. 37% and 27%), cell number of each blastocyst on Day 8 (135 ± 4.1 vs. 116 ± 3.6, and 101 ± 4.2), and the percentage of Grade A blastocysts (54% vs. 42% and 29%) in the hybrid group were higher than in the Holstein and Yellow groups. The proportion of blastocyst production on Day 7 was greater in the hybrid group than in the Holstein and Yellow groups (89% vs. 71% and 63%). The blastocyst rate from morula in the hybrid group (84%, 37/44) was significantly higher than in the Holstein group (63%, 52/83) and the Yellow group (59%, 24/43). Taken together, these results strongly show that hybrid recipient oocytes can improve the development of cloned bovine embryos. It is suggested that the genetic heterogeneity of the hybrid recipient cytoplasm would lead to more possibilities of reprogramming and embryo development.

Table 1. Effect of different recipient oocytes on development of cloned embryos

Parameters	Hybrid	Holstein	Yellow	Total
No. of matured oocytes	256	614	238	1108
Fused reconstructed embryos (%)	110 (43)[a]	216 (35)[b]	120 (50)[a]	446 (40)
Cleavage (%)	73 (66)	142 (66)	90 (75)	305 (68)
Blastocysts (%)	37 (51)[a]	52 (37)[b]	24 (27)[b]	113 (37)
Cell number of blastocysts (Day 8)	135 ± 4.1[a]	116 ± 3.6[b]	101 ± 4.2[c]	118 ± 3.1

[a–c] Values with different superscripts within the same row are significantly different ($P < 0.05$).
Data were analyzed by ANOVA and chi-square using SAS (SAS Institute, Inc., Cary, NC, USA).

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Embryo Transfer

158 PREGNANCY RATES IN RECIPIENT COWS TREATED WITH PROGESTERONE VAGINAL DEVICES AND INDUCED TO OVULATE WITH ESTRADIOL BENZOATE GIVEN AT THE TIME OF DEVICE REMOVAL OR 24 h LATER

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Although treatments with progesterone (P4) releasing devices, estradiol benzoate (EB) and eCG have been shown to result in acceptable pregnancy rates after embryo transfer, the treatment requires that the cows be run through the chute at least four times for treatments. An experiment was