BOVINE IN VITRO FERTILIZATION AND CLONING FOR BEEF AND MILK PRODUCTION

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Abstract

Technical feasibility has been adequately demonstrated to lend encouragement to international trade of bovine embryos resulting from in vitro fertilization (IVF). Major barriers to widespread implementation of IVF technology have included oversized calves, dystocias, elevated stillbirths and neonatal deaths primarily following embryo transfer (ET) of fresh in vitro produced (IVP) embryos since successful cryopreservation reflected in acceptable pregnancy rates has not been widely demonstrated. The proclivity of oocytes and resulting IVP embryos to adsorb viruses not easily removed by washing procedures established as efficacious for in vivo embryos represents another cause for concern. In addition to IVF, bovine embryos can be produced by initiating embryonic development of enucleated oocytes using skin fibroblast or granulosa cell nuclei to replace the genetic blueprints normally provided by the gametes. Although cloning by nuclear transfer (NT) has been demonstrated to result in calves by several groups current NT procedures require large numbers of oocytes since very low proportions result in IVP embryos and more importantly, pregnancy losses, dystocias, oversized offspring and neonatal deaths represent formidable problems to be resolved. Expenses incurred in production and maintenance of NT calves can predictably limit early impact of cloning apart from production and expansion of highly valued transgenic cattle employed for production of biological products for world markets. By contrast, it seems likely that ET of IVP embryos resulting from IVF can supersede artificial insemination to enhance world-wide reproduction of desirable beef and dairy cattle in the near future.

Introduction

More people were added to the world's population in the past 50 years than in the preceding million years. Although a variety of factors have led to slowing in human population growth in the past decade, world population is projected to grow from around 6 billion currently to about 9.4 billion by 2050 (medium fertility assumption) with aging emerging as the most pressing demographic issue facing humanity early in the new millennium(1). Doubling, and even tripling, of food supplies will be required to sustain anticipated populations in many, especially developing, countries.

Animal biotechnology is poised to contribute to the world's nutritional requirements. As artificial insemination(AI), embryo transfer(ET), and cryopreservation methodologies have advanced efficiency in food production through improved cattle breeding during recent decades newer approaches promise even greater impact for the future. Since the first calf resulted from in vitro fertilization (IVF) in 1981 (2,3) continuing progress in research has moved this technology from the laboratory into the commercial arena. Anticipated developments should position bovine IVF in combination with ET as the next generation technology of choice for pregnancy initiation. Thus, ET of embryos produced in laboratory conditions can be predicted to supersede AI as the most efficient means for establishing pregnancies in cows during the coming years.

Also, during the past two decades identical lambs (4) and calves (5) were shown to result from totipotent blastomeres separated from early cleavage stage embryos prior to ET into separate recipients for development. Initial steps toward large-scale cloning of domestic animals by nuclear transfer (NT) began in 1984 when a sheep was produced by combining a single blastomere from an 8-cell stage embryo with the enucleated half of an unfertilized egg (6) and the methodology was extended to cattle (7). The birth of Dolly derived from ET of a blastocyst that developed after NT of a mammary gland cell nucleus from a 6 year old ewe into an enucleated sheep oocyte (8) opened the door to cloning by NT using differentiated somatic cells as nuclear donors. This potentially powerful new technology holds much current promise

for use in expediting production of transgenic founders and their rapid propagation to establish production herds of transgenic cattle. By contrast to IVF, NT technology as a widely useful production methodology must await improvements in efficiency with which such procedures can be successfully carried out. This paper is intended to briefly present the state-of-the-art with respect to IVF and cloning technologies in cattle along with thoughts on their current and near-future potential applications.

In Vitro Production (IVP) of Bovine Embryos

Progress in bovine IVF technology during the past two decades has followed intensive investigations of the physiological events, e.g. oocyte maturation, fertilization, early embryonic development, and exploration of ways to integrate IVF along with other new technologies, e.g. cryopreservation, sexing, ET, into production systems for commercial use. The two lines of research are obviously interdependent since commercialization cannot be realized in absence of a reasonable degree of success in producing embryos (esp. blastocysts) from oocytes after in vitro insemination and culture and such in vitro produced, or IVP, embryos must be able to withstand certain rigors that in vivo (flushed) embryos are subjected to in order to effectively develop following ET. For practical purposes reports demonstrating improvements in IVF results, and especially those linking laboratory IVP embryo production with live calf production are of greatest interest. Progress along these lines is illustrated by several examples (summarized below) from currently available literature.

Bovine In Vitro Fertilization and Embryo Transfer

Currently, IVF is being used when valuable donor cows are no longer producing high quality embryos in sufficient quantities by conventional ET approaches. Bousquet et al. (9) reported data acquired over a 3 year interval demonstrating that IVF procedures can effectively replace conventional in vivo embryo production methods when a predetermined number of pregnancies of known sex are needed within a short period of time. Oocyte donors were mature (7 to 14 year old) nonlactating dairy cows (n=92) maintained in the L'Alliance Boviteq ET center in Canada. Ultrasound guided transvaginal ovum pick-up (OPU) following 48h pre-treatment with FSH provided 4145 oocytes from 437 oocyte recovery sessions (9 to 10 oocytes per session on average). Results (summarized with mean values) from their IVF protocol showed that 4 oocyte collections per cow performed within a 60d period yielded 38 oocytes which, in turn, resulted in 18.8 viable embryos (49.5% blastocysts from oocytes). All excellent and good quality (10) embryos were biopsied and sexed by a PCR procedure, (11) and 7.05 of the 18.8 were females. After ET of the female embryos an average of 3.8 recipients were pregnant at 60d (53.9% pregnancy rate after ET of fresh biopsied embryos). By comparison, 1652 ova and embryos were flushed from 49 cows in 156 conventional embryo collections. One collection of in vivo embryos yielded 1.2 female pregnancies confirmed during the same 60d time period. Thus, in spite of the higher male to female ratio (1.5 to 1 in favor of males) and higher embryonic mortality (6.7% higher in this study, 9.6 vs 2.9%) the number of confirmed female pregnancies was still over 3 times higher using the IVF approach (9). In this work, a herd of 200 heifers were maintained as recipients for the ET of fresh (never frozen) embryos. The oocyte donor cows for IVF were formerly in a regular embryo collection program but were transferred to the IVF program because they were producing non fertilized ova or because their oviducts were blocked. Oocyte maturation was completed in a fetal calf serum-containing medium and IVC was in serum- supplemented medium supporting co-culture with oviductal cells up to day 8 post-insemination.

Sirard et al. (12) showed a 48h period of FSH starvation between the last injection and oocyte recovery to be beneficial for IVF. An extension of this work involved 8 first lactation Holstein cows treated or not treated with LH (25mg,iv) 6h before oocyte recovery (OPU) with 30d intervals between cow treatments (13). The LH treatment had no positive effect on overall embryo production; 52% of oocytes developed into transferable quality (grade 1 and 2) embryos by day 8 in their IVP protocol with or without LH and with 33 or 48h of FSH starvation before OPU. However, LH had a tendency to improve the competence of oocytes collected from large follicles after 33h of FSH starvation. Follicles $\geq$ 5mm tended to yield more competent oocytes than smaller ones. Thus, removal of the donor cow's dominant follicle (>6mm diameter) and beginning 2 days later, i.e. between estrous cycle days 8 and 14, treatment for 2d with 4 injections of 50 mg FSH ("Folltropin", Vetrepharm, Canada) followed by LH treatment 27 or 42h after the last FSH treatment with oocytes recovered from >5 mm follicles by the transvaginal guided approach 6h later yielded a total of 89 oocytes of which 81 (91%) cleaved and 54 (61%) developed into transferable blastocysts (13).

In 1998 (the most recent year of record) only 164 pregnancies were reported to result from ET of frozen IVF

embryos worldwide (14). Economic advantages of embryo cryopreservation include eliminating the need to maintain a herd of recipient cows, i.e. rather, use of recipients managed and synchronized on farms, and the increased potential for transferring every embryo. Additional efficiency is offered by embryo sexing (or sex predetermination) to allow production of only calves of the desired sex. Usually, more rapidly growing males are wanted for beef, and females are essential for dairy herds. Data on biopsied, sexed and cryopreserved IVP bovine embryos and their in vivo developmental competence are limited. Agca et al. (15) reported 90d pregnancy rates of 44% (7/16), 23% (3/13) and 50% (7/14) for vitrified, frozen and fresh embryos, respectively ($P<0.10$). Subsequently, results with vitrified IVP embryos after biopsy and sexing (by DNA fingerprinting) included a pregnancy rate of 40% (16/40) with single embryo transfer compared with 65% (20/31) for AI ($P<0.05$). All AI fetuses developed to term but only one-half of those from single ET of IVP embryos were carried to term (8 calves from 16 fetuses). Combination of AI and single embryo transfer resulted in a pregnancy rate (day 40) of 75%, 27/36 with 11 carrying twins and the pregnancy rate after bilateral transfer of two embryos was 72%, 26/36 with 10 carrying twins. Calving rates for the latter two groups were 76 and 70% respectively, of those pregnant at day 40 (i.e. 50% calving rate when 2 embryos were transferred to each recipient). Mean birth weight and dystocia score for single-born calves were greater than those of twin-born calves ($P<0.05$). Although Agca et al. (15) showed that normal calving and offspring could occur after biopsy and vitrification, their report also serves to emphasize a need for technical improvements. Thus, of 5964 oocytes matured and fertilized 1318 developed to the morula stage (22%) at day 5 (in vitro insemination = day 0). Of these, 623 were biopsied and 359 (58% of morulae biopsied) developed to blastocysts and were vitrified (approx. 13% development of oocytes to blastocysts); 158 were female, 139 males and 62 undetermined after the sexing assay. Of 158 female embryos thawed 148 were transferred (single and double transfer groups) and 34 calves resulted (i.e. approx. 23% of embryos transferred, representing only around 3% of oocytes used), but 5 of these were male reflecting accuracy in the sexing procedure of 85% (29/34). These results suggest double embryo transfer of sexed embryos to be more desirable since 36% of 72 embryos transferred in this group went to term while only 20% of 40 single embryos transferred survived to birth. The distinct possibility of initiating twin pregnancies with transfer of two embryos emphasizes the desirability of sexing to avoid freemartins resulting from simultaneous gestational development of male and female conceptuses. This may be mandatory in non-beef production systems.

Production of bovine embryos of known sex combined with cryopreservation and successful outcome following ET will become commercially applicable. The use of sexed sperm cells for IVF promises an attractive alternative to currently employed sexing requiring removal of embryonic cells by biopsy. Recent improvements in sperm cell sorting (16) make it possible to sort around 10×10^6 sexed sperm/h. Cran et al. (17) produced blastocysts from 17% of oocytes inseminated with sexed sperm and 106 twin transfers resulted in birth of 37 males and 4 females (90% efficiency in production of the desired sex). More recently, Hamano et al. (18) reported the birth of 10 calves from sorted, injected sperm with 80% efficiency in producing the desired sex. Sorted sperm provide cleavage rates comparable to unsorted sperm but blastocyst development is impaired (19, 20). Lu et al. (20) reported similar blastocyst development (around 70% of the rate with non-sorted sperm) with sorted fresh, and sorted, then frozen sperm. In this work fertilization and culture were in semi-defined conditions and use of sorted or non-sorted sperm for insemination in drops or in straws yielded 16 to 21% blastocyst development from oocytes (20). Lu and Seidel (21) reported in vitro maturation (IVM) in M199 + 10% estrous cow serum + 2 million granulosa cells/ml, sexed sperm insemination in citrate and EDTA-containing defined medium and IVC after the first 48h in the same medium with essential amino acids, no EDTA, but supplemented with 0.12 iu/ml insulin to improve results. Cleavage of 74%, and blastocyst development of 21.8% of oocytes resulted, and 62.4% of the day 7 blastocysts were grade 1 to 2.

Abortion rates following ET of IVP embryos have been reported to be in the 24 to 50% range (15, 22, 23). Calving rates following single transfers of intact fresh IVP embryos have been reported to be 35% (23) and 42% (24). Calving rates defined as number of calves per recipient of about 70% have been achieved with ET of two fresh IVP embryos (25, 26, 27, 28). Kajihara et al. (28) reported 50% calving rates following transfers of two frozen embryos into each recipient..

Calf mortality rates frequently associated with excessive birth weights and dystocias (in as many as 60% of recipients) have been reported to be as high as 50% (29). Schmidt et al. (30) reported 38% perinatal mortality of IVP twin calves. Among their postmortem findings were cerebellar hypoplasia, pneumonia, enteritis and multiple muscle necrosis. Agca et al. (15) reported less than 4% (2/62) neonatal mortality, with no malformations, which is close to the 9% calf mortality rate reported after ET of in vivo derived embryos (31). As for mortality in calves resulting from natural matings

(32) excessive birth weight is a major cause of death in IVP calves. Farin and Farin (33) found their IVP calves to be 17% heavier than in vivo produced controls at 7 months gestation. Hasler et al. (23) found that 7% of their IVP calves weighed over 151 pounds. In reports where the same semen was used for AI and for IVP embryos the birth weights of IVF-derived calves were 7% (34) and 16% (24) heavier than those resulting from AI. Longer gestations have been reported for IVF-derived calves than for those resulting from AI (24, 34), although this is not always the case (15). Gestation length for twin-carrying recipients is normally shorter than that of singles (22).

Some data have been interpreted to suggest that IVP embryos from non co-culture conditions do not result in oversized calves (35). Recent data of Behboodi et al. (36) implicate IVM and IVF conditions as causes of higher birth weights of live calves resulting from ET of morulae and blastocysts that developed in coculture with BRL cells in B2 medium containing 10% fetal calf serum after microinjection of in vivo and in vitro fertilized ova. Embryo development to morulae or blastocysts was significantly higher in the in vivo-derived ova, i.e. 19% vs. 9% ($P<0.05$). Rates of development to live calves after ET of singly transferred morulae and blastocysts originally matured and fertilized in vitro and in vivo were 17.1% and 21.8% respectively and corresponding birth weights were 45.4 and 42.2 kg ($P<0.05$). All calves were delivered by cesarean section.

Agca et al. (35) reported around 90% re-expansion of day 7 blastocysts at 24h and hatching of 76% at 72h post thaw following a vitrification protocol including cryoprotection afforded by 5 min exposure to ethylene glycol at 27°C. After this vitrification procedure 27% of 11 recipients receiving single unselected embryos, and 60% of 15 recipients receiving morphologically-selected embryos became pregnant but the animals were not followed beyond 120 days. This work was extended to involve comparison of pregnancy, calving and calf survival rates after bilateral ET of vitrified, conventionally frozen and fresh embryos. They reported no significant differences in day 40 pregnancy rates between fresh and vitrified embryos, fresh day 6: 31 of 40 (78%), fresh day 7: 24 of 35 (68%); vitrified: 10 of 16 (63%) but the twinning rate was significantly lower for vitrified embryos compared to fresh embryos, 2 of 10 (20%) vs. 16 (52%) of 31 pregnancies for fresh day 6, and 14 (60%) of 24 pregnancies for fresh day 7 embryos. The day 40 pregnancy rate was significantly lower for conventionally frozen IVP embryos (7 of 17 or 41%) and the twin pregnancy rate at 120 days for this group was similar to that following ET of vitrified IVP embryos, i.e. 2 of 7 pregnancies (29%). Subsequent to 120 days, abortions of 5, 3, 4 and 2 fetuses were reported for the day 6 fresh, day 7 fresh, vitrified and conventionally frozen treatments, respectively. In addition, seven twin-born calves and one single-born calf died at birth or shortly thereafter. Among these losses one set of twins was born prematurely at 250 d and two fetuses were oversized (114 and 105 lbs) while the other losses were completely unexplained. According to groups the calf losses were 3 each for fresh day 6 and day 7 groups and 1 each for the cryopreserved IVP embryo groups. Close scrutiny of these bilateral ET data reveals that selected IVP blastocysts of grade 1 (good and excellent quality) transferred fresh on day 6, transferred fresh on day 7, vitrified before ET, and conventionally frozen before ET resulted in 48.8% (39/80), 45.7% (32/70), 21.9% (7/32), and 17.6% (6/34) development to live (surviving) calves, respectively. Thus results of Agca et al. (35) were comparable to earlier reports by Kajihara et al. (28) of 60% pregnancy rates following vitrification and single transfers of only post-thaw surviving embryos, and of 23% pregnancy rates by Reinders et al. (37) and Wurth et al. (38) after ET of IVP embryos not selected post-thaw. Although abortion rates, 9% after ET of fresh embryos, 28% after ET of cryopreserved embryos (35) are apparently lower when two embryos are bilaterally transferred, i.e. into different uterine horns, compared with those, 20 to 45% (26), resulting from unilateral transfer of two embryos, i.e. into the same (ipsilateral to the CL) uterine horn, such losses can hardly be considered acceptable.

Efforts to Improve Bovine In Vitro Fertilization

As long as viability of IVP embryos is less than that of in vivo embryos as determined by ET, researchers will continue efforts to improve media and other conditions to better support IVM, IVF, and IVC. Lazzari et al. (39) reported temporal enhancement of blastocyst development by including 10ug/ml heparin for IVC in SOF supplemented with BSA and non-essential amino acids (NEAAs). Although comparable proportions of oocytes reached the b1astocyst stage on day 8 (40 and 42% of cleaved ova, without and with heparin for IVC, respectively) significantly more blastocysts were formed on day 7 when heparin was present, i.e. 32 vs. 23 % ($P<0.05$). Cell numbers of day 7 blastocysts were higher in the group cultured without heparin and cell numbers of those cultured with heparin were similar to cell numbers of bovine blastocysts resulting from in vivo culture in sheep oviducts. Lee et al. (40) demonstrated improvement in proportions of blastocyst

development from 31.7% to 49.4% of the cleaved oocytes by addition of Beta- mercaptoethanol (12.5µM) to KSOM + 10% FCS for IVC. Lazzari et al. (41) found 60% and 24% of the blastocysts that developed by day 7 were derived from embryos that were compacted morulae on day 5 or day 6, respectively. Around half of the blastocysts forming on day 8 were from embryos that did not compact at all. Since blastocysts forming on day 8 are less viable (23) and contain lower cell numbers than those forming on day 7 it was suggested that selection of compacted morulae on days 5 and 6 might increase accuracy of selecting embryos for transfer or cryopreservation (41).

Culture of individual or few embryos becomes necessary when oocytes are collected by ultrasound-guided transvaginal recovery techniques. Defined culture conditions are preferable for allowing better standardization and reducing risk of contamination from undefined biological material. A novel system for individual embryo culture (Well-of-Well, WOW) employs small conical and cylindrical wells (WOWs) approximately 300 µm deep made with heated iron rods in four well Nunc plastic dishes (42). After washing overnight and rigorously rinsing WOWs were filled with 400µl of a modified SOFaa medium covered with an equal volume of paraffin oil. The WOW system enabled Vajta et al. (42) to achieve (day 7) blastocyst per oocyte (presumptive zygote) rates of 59%, 61% and 53% for single embryos, group of 5, and single zona-free embryos, respectively, and these rates were higher for all than achieved in cultures in drops or in wells. Culture of single embryos in the Well-of-Well culture system to the blastocyst stage can be achieved in modified SOF, i.e. SOFaaci (43) with PVA (defined IVC medium) but an additional one-half to one day is required when compared with culture in the presence of 5% bovine serum (44). The proportions of zygotes reaching blastocysts decreased from 48.9% to 40.8% ($P<0.05$) when cultured in groups of around 20 per 400µl with serum compared to the same conditions with PVA replacing serum, and from 49.3% to 30.7% ($P<0.05$) when cultured singly with serum compared to those cultured singly with PVA replacing serum (44).

Long et al. (45) compared influences of three semi-defined media on embryo development after IVF of postmortem oocytes. Development rates (to day 7 blastocysts) were similar for groups in BARC-1 (modified synthetic oviductal fluid developed by K. Wells) and G1.2/G2.2 (46) and significantly ($P<0.01$) higher than CR1aa (47); embryo development rates were 12.2, 14.9 and 1.0%, respectively. Subsequently CR1aa supplemented with 10% fetal bovine serum on Buffalo rat liver (BRL) cell co-culture, BARC-1, and G1.2/2.2 were compared in a commercial IVP lab using a total of 1,542 oocytes from 172 aspiration sessions on beef cattle of various breeds and ages. Endpoints reported were proportions of oocyte development to blastocysts and proportions of embryos transferred that resulted in pregnancies at 60 days. Corresponding data were 17.4% and 56.8%, 19.7% and 45.2%, 19.9% and 64.5% for CR1aa/BRL, BARC-1, and G1.2/G2.2, respectively. With the exception of the BARC-1 group there were no significant differences in pregnancy rates afforded by the lab-grown embryos and 61.8% resulting from coventional ET of 401 in vivo embryos (45).

Hasler et al. (48) compared efficacy of G1.2/G2.2 media (47) with co-culture comprised of B2 medium supplemented with 10% serum on BRL cells for blastocyst development after IVM and IVF involving 2,945 oocytes. In the coculture system 26.7% of oocytes reached the blastocyst stage on day 7 compared to 21.4% for oocytes cultured in G1.2/G2.2; however, cell numbers were 207 after culture in G1.2/G2.2 vs. 169 after coculture ($P<0.05$) and inner cell mass cell numbers were also higher for G1.2/G2.2, i.e. 82 vs. 63. Post-thaw survival rates of cryopreserved blastocysts were not different. Three of 5 frozen/thawed blastocysts transferred from the G1.2/G2.2 group established on-going pregnancies.

Completely Defined Conditions for IVP of Bovine Embryos

Employment of completely chemically defined media affords an opportunity for improvement and further refinements of chemical and physical conditions to enhance efficiency of IVP. Defined media for complete support of immature oocyte to blastocyst development are now available (43,49,50,51,52). Concern surrounds the possibility of disease transmission via viruses (from oocyte donors, co-culture cells, serum, or any fluids of biological origin) that can become adherent to zonae pellucidae of IVP embryos (53). Pathogens as BVDV (54), BHV-1 (55) and BTV (56) become much more closely associated with the zonae of IVP embryos and are not completely removed by the standard washing and trypsin treatment recommended for in vivo embryos by IETS (57). In our laboratory both a non-specific protease treatment (58) and a photoinactivation treatment (59) used earlier in experiments with in vivo embryos (60) were found to be encouraging candidates for virus removal/inactivation but further efforts are warranted.

In our completely defined IVP system (49,51,52) 55% of unselected oocytes recovered postmortem from ovarian follicles of $\leq$ 5 mm diameter undergo IVF and cleavage to the 4-cell stage and 30% of these (17% of oocytes) develop into blastocysts of transferable quality. Substantial improvement in IVP results, i.e. proportion of blastocysts from oocytes, can be obtained when these conditions are applied to oocytes that are morphologically selected (61) for potential ongoing viability (49). Advantages in not selecting oocytes include time savings, potentially more embryos and greater repeatability by different technicians. Only a few calves have been obtained from ET after IVP in completely defined conditions. In the author's laboratory procedures for IVP of bovine embryos in chemically defined conditions combined with cryopreservation (62) and direct single ET into 12 recipients resulted in the birth of four calves including one stillbirth (unpublished).

Cloning

Embryo Cloning

Several approaches to cloning have resulted in identical calves. During the past two decades embryo splitting (63) has been offered as a commercial option by many embryo transfer organizations. By this means desirable calf production commonly represented more than 100% of original embryos recovered by flushing uteri of donor cows. Separation of blastomeres also offers the means for production of identical multiplets and this approach has been successfully applied to demonstrate blastomere totipotency in cattle (5). Commercialization of this technology has not been exploited and the degree of technical difficulty may preclude any immediate widespread applications. The most attractive approach to embryo cloning has been by nuclear transfer and although procedures were adequately developed to make it commercially available a decade ago the gestational calving and neonatal difficulties proved formidable. Additionally, sexual reproduction makes uncertain the potential value of embryos as adult cows two or three years later. In spite of this earlier experience there seems to be a revival of interest in at least developing the technology to enable widespread cloning of bovine embryos by NT (64).

Cloning by Somatic Cell Nuclear Transfer (NT)

By 1996, Campbell et al. (65) had developed the capability of cloning sheep by NT from a cultured cell line. Extension of this work with additional embryonic cells, differentiated fetal and adult cells resulted in offspring following NT from each of the three cell types (8). Success in cloning animals from transfer of nuclei from adult cells has been attributed to quiescence of the donor cells and such cells are induced to leave the growth cell cycle and become quiescent by serum starvation. In 1998, Cibelli et al. (66) produced calves from NT of fetal fibroblasts that were earlier altered to carry a transgenic marker and that were reported not to be quiescent, but in the G1 phase of the cycle. Successful NT from somatic cells has recently followed experimentation in laboratory and domestic animal species and the controversy regarding use of nuclei from cells in G0 or G1 continues (67).

Another approach involves use of bovine oocytes in which the level of MPF is reduced by aging at reduced temperatures (68). This environment allows the nuclear envelop of the transferred nucleus to remain intact presumably until the nucleus itself determines its DNA replication. Calves have been produced using this means for coordinating the cell cycle (69).

Kato et al. (70) produced eight calves from NT of quiescent cumulus cells from a donor cow. In their work nuclei were fused to non-activated cytoplasts (i.e. enucleated metaphase II oocytes). The report of Wells et al. (1971) serves to illustrate the state-of-the-art in cattle cloning by NT. Cultured mural granulosa cells obtained from a Friesian dairy cow of high genetic merit were the source of nuclei. Quiescent cultured cells were electrically fused to enucleated metaphase II cytoplasts 4 to 6 h before activation (fusion before activation, FBA treatment). Also, some first generation morulae were recloned by fusing blastomeres to S-phase cytoplasts. On day 7, 27.5% blastocyst development after FBA resulted from fused embryos but only 13% for recloned embryos. After ET of 100 blastocysts after FBA treatment, successive survival rates for days 60, 100, 180 and term were 45%, 21%, 17% and 10%, respectively. Ten heifer calves were delivered by Cesarean section , all survived, and they were confirmed to be genetically identical to the donor by DNA analyses. Embryo survival of 16 recloned blastocysts on day 60 was 38% but no fetuses survived to day 100. Placental dysfunction at specific stages was thought to play a role in the losses.

Wilmut et al. (67) recently described the state-of-the-art as follows. Although repeatable, current NT procedures are very inefficient with only 1 to 2% of reconstructed embryos surviving to become live offspring (67). Far greater than

normal pre-natal mortality occurs throughout gestation and greater perinatal mortality associated with congenital abnormalities and oversized offspring are characteristic results. Further research is required to understand how a transferred nucleus reprograms gene expression and causes of failure. Anticipated new information from intensive research in a variety of animal species should enhance proportions of cloned embryos that can develop into normal identical calves.

Obviously it would be desirable to have multiple copies of cattle that exhibit the highest production traits for meat and milk production, or of the best bulls known for siring offspring with extraordinary production traits. Application of cloning by NT in selective breeding schemes is not practicable at present due to the low efficiency for producing normal calves (72). The potential exists for dissemination of superior genetic livestock in the future by judicious use of NT. It will always be important to maintain genetic diversity, resulting from sexual reproduction, without which large populations of identical cattle could easily be lost to a single devastating disease.

Practical Considerations

Although the economics do not yet provide encouragement for implementing NT for producing the conventional commodities associated with cattle, new opportunities for genetic modification are clearly presented. Genetic manipulations of cells in culture combined with NT now make possible rapid genetic modifications in cattle that could only be done previously in mice (73). Thus, as a reflection of the estimated value of certain protein products in demand as pharmaceuticals or for industrial purposes, monies of several commercial organizations are supporting NT efforts to produce transgenic cattle to supply valuable protein products in their milk. Subsequent application of NT procedures can then be useful in cloning transgenic founder animals to increase the capacity for producing the desired product. Polejaeva and Campbell (73) include as candidates for gene targeting: replacing bovine serum albumin with the human form to allow cost-effective production in milk of cows; and knockout of the Prion gene involved in bovine spongiform encephalopathy (BSE). With identification and characterization of other genes important for production, disease resistance, etc., additional avenues for genetic modification of cattle will surely be traveled.

By contrast, IVF technology is already finding utility in cattle breeding as described above. Continuing research to improve media to support IVP of bovine embryos should result in improved quality and resulting gestational viability. Such progress can be predicted to make IVP bovine embryos more attractive economically. Additionally, improvements in IVF technology can be anticipated to find utility in further development and commercialization of NT technologies.

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Keywords

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