IMPACT OF ADVANCED REPRODUCTIVE BIOTECHNOLOGIES ON ANIMAL HEALTH AND LIVESTOCK PRODUCTION

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ABSTRACT

The *in vitro* production of bovine embryos by *in vitro* fertilization or nuclear transfer procedures is a powerful tool in wide use for scientific, conservationist and commercial purposes. However, developmental abnormalities are unpredictable consequences of such *in vitro* embryo manipulations, which may interfere with the pattern of fetal and placental growth and life *ex utero*, in a set of symptoms collectively called Large Offspring Syndrome (LOS). The economical significance of the syndrome is associated with increased rates of pregnancy losses, placental and fetal aberrations that culminate in abortion, hydrops of the fetal membranes, prolonged gestation, diminished signs of parturition, dystocia, and birth of large calves with lower postnatal survival. Lower pregnancy rates with higher gestational and postnatal losses represent significant economical losses for a lower prolificacy. The understanding of mechanisms of prenatal growth in normal development and in those related to the syndrome will be of significance for prevention or attenuation of abnormalities of common occurrence in cattle, with potential direct scientific and economical implications.

Keywords: LOS, large offspring syndrome, *in vitro* produced embryos

EL IMPACTO DE LA BIOTECNOLOGÍA REPRODUCTIVA AVANZADA EN LA SALUD Y LA PRODUCCIÓN ANIMAL

RESUMEN

La producción *in vitro* de embriones por fertilización *in vitro* o por procedimientos de transferencia de núcleos es una herramienta poderosa utilizada ampliamente para propósitos científicos, conservacionistas y comerciales. Sin embargo, las alteraciones del desarrollo son consecuencias impredecibles de tales manipulaciones *in vitro*, que pueden interferir con el patrón de de crecimiento fetal y placentario y la vida *ex utero*, que se agrupan en unos síntomas conocidos colectivamente como el síndrome del ternero grande (LOS). La importancia económica del síndrome está asociada con un incremento en las pérdidas gestacionales, aberraciones placentarias y fetales que culminan en aborto, condiciones hidópicas de las membranas fetales, gestaciones prolongadas, disminución en los signos de parto, distocias, y nacimiento de terneros grandes con baja supervivencia postnatal. Las bajas tasas de preñez con las altas tasas de pérdidas gestacionales representan pérdidas económicas significativas por baja prolificidad. El entendimiento de los mecanismos de crecimiento prenatal durante el desarrollo normal y en aquellos relacionados con el síndrome sería muy significativo en la prevención, atenuación de las anormalidades de ocurrencia común en el ganado, con implicaciones directas a nivel científico y económico.

Palabras clave: LOS, syndrome del ternero grande, embriones producidos *in vitro*

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INTRODUCTION

It has been more than two decades since the birth of the first bovine calf (Virgil) from *in vitro* fertilization (IVF) procedures (1), and the first cloned mammal (sheep) by nuclear-transfer (NT) technology using blastomeres from preimplantation-stage embryos (2). In those days, bovine embryos were usually produced using *in vivo*-matured oocytes and, after IVF, early cleavage-stage zygotes were *in vivo*-cultured to the morula or blastocyst stage in the oviducts of surrogate recipient animals, usually the female sheep or rabbit. Today, the *in vitro* production (IVP) of embryos from IVF or NT procedures for bovine embryos consists of three steps: first, *in vitro* maturation (IVM) of primary, germinal vesicle-stage oocytes collected directly from the ovaries of donor females; second, IVF by combining *in vitro*-matured oocytes with *in vitro* capacitated sperm cells, or NT by fusing a somatic cell to an enucleated oocyte; and third, *in vitro* culture (IVC) of presumptive zygotes for a period of 6-10 d, when embryos reach stages of development that allow them to be transferred to female recipients. The birth of Dolly in July of 1996 by transfer of a somatic-cell nucleus from an adult ewe into an enucleated oocyte (3) represented the height of such advancement in IVP technology.

Cloning and IVF technologies became a widely used biological tool for scientific, conservation, and/or commercial purposes. The technology of IVP of bovine embryos has shown a remarkable increase in efficiency and use over the past few years, with the transfer of IVF-derived embryos representing more than 30% of the total worldwide bovine embryo transfer activity in 2005 (4). Many advantages have been identified for the use of this technology over conventional embryo production systems, and several scientific and commercial applications have also been associated with cloning by SCNT, including reproductive cloning, for the conservation and propagation of economically important individuals and endangered animals, and therapeutic cloning, which may eventually have a direct impact on human health. However, pre- and postnatal deviations in development became unexpected and unpredictable consequences of such *in vitro* manipulations, and have become an important animal welfare issue, also limiting the transfer of such modern reproductive technologies into commercial agricultural and biotechnology practice. Such events occurring during the first days after IVF or embryo reconstruction by NT may interfere with embryonic, fetal, and placental development *in utero*, in a set of events commonly referred to as Large Offspring Syndrome (LOS).

THE LARGE OFFSPRING SYNDROME

In their comprehensive and inspiring review, and making use of scarce material, Walker et ál. (5) hypothesized a series of mechanisms to explain the appearance of abnormalities following *in vitro* embryo manipulations. Some of their suggestions have since been discounted, whereas others expanded in importance, as new pieces of evidence accumulated. The problem of abnormally large offspring was first described for calves born from blastomere NT procedures (6, 7) but was subsequently shown to occur in embryos produced by IVF (8, 9) and SCNT (10, 11, 12, 13) procedures. The occurrence of LOS appears to be intrinsic to the conceptus, having a worldwide distribution, with most laboratories producing IVP embryos having experienced the phenomenon. Not all offspring derived from manipulated embryos, including identical clones, are affected, demonstrating its epigenetic origin. In addition, the extent of

changes can vary between individual embryos in the same culture, between NT and IVF protocols, or between laboratories (14, 15, 16). The significance of the phenomenon is manifested by fetal and placental abnormalities that are associated with increased rates of pregnancy losses, particularly in the first 60 days of gestation (11, 17, 18, 19), and placental and fetal alterations that culminate in hydrops of the fetal membranes, late abortion, prolonged gestation, diminished signs of parturition, dystocia and birth of large calves with lower postnatal survival (5, 7, 8, 10, 11, 12, 13, 20, 21, 22, 23, 24, 25, 26). Because of the multitude of symptoms related to the syndrome, and due to a placenta-cause fetal-effect mechanism for the appearance of abnormal phenotypes, authors even have recently suggested the use of "Cloning Syndrome" (21), "Abnormal Offspring Syndrome" (26) or even "Large Placenta Syndrome" (25) as more appropriate terms to describe the phenomenon.

DEVELOPMENTAL PROBLEMS DURING PREGNANCY AFTER *IN VITRO* EMBRYO MANIPULATIONS

Currently, a wide range of conceptus abnormalities have been described at the morphological (macroscopic and histological), physiological (functional and metabolic) and/or molecular (epigenetic and gene expression) levels at most developmental periods for IVF- and SCNT-derived pregnancies. To facilitate research efforts towards the understanding of the phenomenon, Farin et ál. (26) introduced a new classification system for IVP pregnancies based on developmental outcomes of the various abnormal phenotypes, according to the degree of abnormalities: Type I, occurrence of severe abnormalities, characterized by early or late embryonic mortality (failure at distinct stages of the embryonic phase); Type II, characterized by conceptus death and abortion during the fetal phase; Type III, abnormalities compatible with a term delivery, but that inflict peri-natal death; and Type IV, moderate abnormalities compatible with a term delivery and normal or abnormal postnatal survival. Since a relatively low number of cloned embryos survive to term (1 to 5%) and approximately a third of cloned calves do not survive to weaning, most phenotypes usually fall within the first three types above. However, since more than 8% die before reaching 4 years of age (21), an additional classification, a Type V phenotype, comprehending the juvenile and adult period should perhaps be included. Pieces of evidence have been suggestive of a placental-cause-fetal-effect on pre- and postnatal survival, as discussed below.

EARLY PREGNANCY: CONCEPTUS GROWTH RETARDATION AND PREGNANCY LOSSES

A limited number of reports investigating IVP concepti during the elongation period have produced some conflicting results. Bovine elongating IVP concepti have been shown to be either more developed on both Days 12 (27) and Day 17 (16) or underdeveloped on Day 16, with shorter trophoblasts and smaller embryonic discs (28). Porcine SCNT-derived concepti have also been shown as underdeveloped on Days 12 and 14, but not on Day 10 (29). Supporting the concept of IVP conceptus underdevelopment, our results suggested the occurrence of a higher rate of early embryonic mortality. Recovery rates and embryonic disc detection for control and IVP concepti on Day 16 were 86 and 56%, and 37 and 35%, respectively (28). Interestingly, pregnancy rates on Days 27-30 for the same pool of IVP embryos, but for the ones allowed to

develop beyond the maternal recognition of pregnancy, were 47% (51/109) and 38% (49/129) for control and IVP embryos (unpublished data), respectively. Such results suggest that a significant proportion of IVP concepti, presumably the smaller ones, were eliminated due to failure to block endometrial $PGF_{2\alpha}$ release at the time of maternal recognition of pregnancy. In addition, IVF- and SCNT-derived embryos or fetuses are smaller at early implantation stages in cattle (30, 31). Consequently, it is logical to conclude that the common expected phenotype for elongating embryos involves underdevelopment of the trophoblast and/or embryonic disc, which may have important implications for the establishment of pregnancy and for the implantation period.

A few reports demonstrated the occurrence of a biphasic conceptus growth pattern in IVP pregnancies. Initially, growth restriction is observed at the end of the embryonic phase and beginning of the fetal phase (28, 31). After the initial period of 'struggle', IVP fetuses that survive seem to experience a period of faster (compensatory) growth after Day 60 of gestation. This phenomenon is preceded by changes in placental development in IVF- (30, 32) and SCNT-derived pregnancies (22), restoring fetal size by the end of the first trimester of pregnancy. Such events culminate either with fetal death or with the delivery of larger IVF and SCNT calves with lower postnatal survival and morphologically altered placentas (19, 22, 30, 32, 33). Under our observations, SCNT-derived late embryos and early fetuses are even more retarded than IVF-derived counterparts (unpublished data).

Failure in developmental events, commonly during the embryonic phase, usually leads to pregnancy losses and significant economical losses in livestock production. Late embryonic mortality in IVP pregnancies represents the major cause for low cloning efficiency (34). In cattle, pregnancy rates at 30 days for embryos derived from IVF or SCNT are usually lower than for embryos produced by superovulation or artificial insemination, but comparable to one another (30-50%). However, gestational losses between Days 30 and 60 of pregnancy are significantly higher after IVP, usually ranging from 15 to 60% for IVF, and 40 and 100% for SCNT (11, 19, 35, 22, 36). At least two mechanistic hypotheses have been used to explain higher embryonic mortality in IVP pregnancies: (a) failure in extraembryonic membrane formation and attachment: suboptimal IVP conditions impair development, leading to conceptus growth retardation, delayed attachment and placentation, further restricting growth, and leading to death for reduced transfer capacity between the embryo and the maternal system (28, 30, 32); and (b) expression of surface antigens from the MHC I complex in the trophoblast of SCNT-derived concepti, which may lead to rejection of the allograft (37). Either possibility is not exclusive in IVP pregnancies.

The phenomenon of biphasic growth pattern is not only limited to the embryo proper or fetus, but also seems to affect membrane development, placentation and embryo survival. In previous studies, placental underdevelopment appears to precede growth restriction or even fetal demise (11, 30). Placentation and organogenesis are the major events occurring during the implantation period, and failures in either or both may be lethal. Observations by ultrasonography from pregnancies that were lost revealed that embryo development seemed to be morphologically normal in shape, but further delayed in size (unpublished data; 31). Underdeveloped trophoblast, precocious regression of the yolk sac and/or a failure of the allantois and vasculature to develop may delay attachment and placentation and, if threshold for survival is not attained, such

events will likely be lethal. Alternatively, Hill et ál. (34) have described compelling evidence demonstrating that rejection of the allograft is linked to increased late embryonic mortality in IVP pregnancies, especially for SCNT. In ruminants, suppression of MHC-I expression coincides with the intimate association between endometrial and chorioallantoic tissues during implantation (34), a period that coincides with a high rate of pregnancy loss in IVP pregnancies. Clone fetuses from Days 34-63 of pregnancy were found to express MHC-I antigens that were not detected in age-matched control pregnancies (34). In addition, a significant maternal lymphocytic response was also detectable in specimens expressing MHC-I peptides, with a close association with growth retardation and fetal death in earlier SCNT-derived pregnancies. It is suggested that MHC-I expression in early pregnancy is caused by faulty genome reprogramming, either by inheritance from the somatic donor cell, by persistent or continuous expression since embryo reconstruction, or by gene activation of MHC-I loci in the trophoblast as development progresses.

PHENOTYPES AT THE FETAL PHASE AND PERINATAL PERIOD

A little more information is available at mid- to late pregnancy, including the perinatal period. This stage is characterized by abortions, high rates of hydrops of the fetal membranes, prolonged gestation and attenuated signs of parturition, dystocia, and birth of large calves with lower postnatal survival.

Conceptus development. Accelerated fetal growth in IVP pregnancies is accompanied by placental enlargement, which precedes the increase in fetal weight in late IVF and SCNT pregnancies (22, 25, 30). Fetal weights are usually significantly higher than controls from mid- to late gestation (22, 25, 30, 38). Our previous studies indicated IVP pregnancies to sustain larger concepti by the second trimester of pregnancy, with a two- to four-fold increase in the accumulation of of glucose and fructose in fetal plasma and associated fluids in comparison with normal pregnancies (32). Likewise, fetal membranes of IVF and placentas of SCNT calves were noted with morphological abnormalities including two-fold increases in surface area and mass with concomitant increases in plasma fructose concentrations in IVF and cloned neonates (23, 32). Consequently, the larger placental mass and surface area in IVF and SCNT pregnancies suggest, at least in part, the existence of increase nutrient supply to the fetus, resulting in increased pattern of prenatal growth that may have implications to postnatal life (32).

Rates of hydroallantois in cattle are <0,1% in normal pregnancies (1/1400 to 1/7500) and <2% in IVP pregnancies (1/55 to 1/200). However, hydrops may vary widely in SCNT pregnancies, ranging from ~0% to approximately 60% (26, 39, 40). Li et ál. (40) detected significant changes in amniotic and allantoic fluid composition in clone and IVF pregnancies. In general, electrolyte homeostasis was compromised to a lesser or greater degree, suggesting problems in fetal kidney function. In contrast, our previous data did not show any significant abnormality in the fetal fluid composition on Days 90 and 180 of gestation, despite the increase in allantoic fluid volume in IVF-derived pregnancies on Day 180 (32). Constant et ál. (25) recently described late gestation placentome morphometry in SCNT pregnancies complicated by hydroallantois, which may provide clues for the understanding of the pathophysiology of this abnormality during pregnancy.

Morphologically and histologically, placentas from IVF- and SCNT-derived

pregnancies are significantly affected throughout gestation, with most obvious aberrations seen during mid- to late pregnancy and at term. Common observations in the third trimester of cattle and sheep IVP pregnancies and at term include placentomegaly, reduced number of placentomes, presence of giant, flattened and/or thinner placentomes, edema, enlarged umbilicus, flattened uterine epithelium, fetal connective tissue enlargement, reduced cell density, reduced vessel dilation and density, immaturity of placental vessels, reduction of villous vascularization and vasculogenesis, among others (22, 30, 32, 33, 38, 25, 41, 42, 43). Authors of a recent comprehensive study have described detailed structure and microvascular architecture distinctions in bovine clone placentas (44), with the most significant ultrastructural findings referred to the presence of dilated caruncular crypts accommodating more than one primary villus with a lack of dense complexes of capillary loops and sinusoidal dilations in clone placentas. Altogether, abnormal vascularization, tissue remodeling, differentiation and maturation of placental tissue in IVP pregnancies may be the primary cause of fetal losses, abnormal fetal organ development, and faulty homeostatic control of organs and systems after birth that may compromise neonatal survival.

Parturition and mammary gland development. Other less characterized events associated with the LOS affect the end of pregnancy and include weaker or absent signals that initiate parturition, which affects the dynamics of the stages of labor, and compromised mammogenesis that seems to influence subsequent lactogenesis. A significant proportion of IVF- and SCNT-derived pregnancies fail to terminate normally, resulting in prolonged gestation. The physiological events linked to parturition appear to be dramatically altered in females carrying IVF- or SCNT-derived concepti. Hill & Chavatte-Palmer (34) attempted to correlate maternal P4 profiles in the last 2 weeks of gestation of SCNT-derived pregnancies with postnatal viability of clone calves. Atypical profiles (no P4 withdrawal pre-partum) appeared to be associated with lower neonatal viability, whereas usual P4 profiles were associated with higher postnatal viability. Matsuzaki & Shiga (45) demonstrated a lower rise in fetal cortisol levels in late SCNT-derived pregnancies, which would not be sufficient to switch the IGF system to a postnatal mode, causing difficulties during parturition. However, little is known about the regulation of these events and how these events are related to failures to initiate parturition and mammary gland development in IVF- and SCNT-derived pregnancies.

Postnatal development. Newborn peri- and postnatal distress and death are very common in cloned calves, frequently associated with problems in adapting to the life *ex utero*, with the frequent occurrence of breathing difficulties, pulmonary hypertension, reluctance to suckle, liver steatosis, abnormal kidney development, longer time to standing and sudden death (5, 10, 20, 21, 24, 34, 46). Some cloned calves seem to fail to exert an effective control over the intermediate energy metabolism during the first 24 h *ex utero*, which is associated with postnatal weakness, hypoxia by pulmonary hypertension, hypo- or hyperthermia, metabolic acidosis and mortality (10, 20, 21, 23, 34). While mean plasma concentrations of many parameters including blood gases, plasma proteins, minerals, and electrolytes are similar in cloned and normal calves, clones are noted with increased variability in these shifted either higher or lower than controls suggesting subtle perturbations of homeostatic mechanisms (23). High neonatal mortality, clinical problems

in the peripartum, and the trend for a reduced lifespan in SCNT-derived calves, with higher morbidity and mortality rates during the juvenile period, have been well described and reviewed by others (10, 12, 13, 21, 47). Loi et ál. (48) have demonstrated that cloned sheep are prone to similar postnatal problems, perhaps even at greater rates than cattle. The authors have linked neonatal respiratory distress and liver and kidney degeneration primarily to placental abnormalities, attaining 100% death rate up to a month of age. Calving difficulty and the frequent need of Caesarean sections in IVF- and SCNT-derived bovine pregnancies also affect the survival and future fertility of the dam, which is translated to additional economical losses.

As more laboratories around the world continue their quest to describe phenotypes throughout development, more information will become available to better clarify the effects of *in vitro* embryo manipulations on short- and long-term postnatal survival. In this view, the implication of the placenta as the major cause of conceptus abnormalities is not unjustified. The placenta is virtually the sole interface responsible for exchanges between fetal and maternal systems, having an important role in fetal growth by the regulation of nutrient supply and synthesis and transport of hormones, substrates, and other substances between systems. In fact, the bioavailability of certain substrates or hormones during pregnancy is important for the establishment of normal patterns of activity of physiological systems in the growing fetus, a phenomenon usually referred as metabolic programming (49). In this theory, changes in the pattern of substrate supply to the fetus may lead to permanent molecular and cellular modifications or even novel patterns of activities in organs and systems that may persist and affect postnatal life. Conditions such as cardiovascular diseases and diabetes originate through cardiovascular, metabolic, or endocrine adaptations that the fetus makes during undernourishment *in utero*, which change fetal programming that may persist for life. The concept of fetal origin to adult's illnesses (50) is based on this phenomenon, which is known as the Barker's Hypothesis (51). Consequently, disturbances in placenta formation due to embryo manipulations may induce changes in the metabolic reprogramming, which in turn may affect placental function. Such changes may lead to growth-related effects, altering events at peripartum, and compromising postnatal survival from birth to adulthood, as predicted by the Barker's hypothesis. Thus, the characterization of each phenotype will help us better understand how environmental or epigenetic changes during the first days in development affect pre- and postnatal life.

MOLECULAR FINGERPRINTS FOR ABNORMAL PHENOTYPES: THE SEARCH CONTINUES

Recently, many reports have stressed the importance of their results on differences in gene expression profiles between IVF-, SCNT- and/or *in vivo*-derived preimplantation stage embryos, either for single genes by real time quantitative RT-PCR or for multiple genes by the way of high throughput approaches, for individual embryos or pools of embryos (52, 53, 54, 55, 56, 57). Such results have demonstrated what everyone already knew for some time: that the embryo production system has a tremendous impact on the pattern of gene expression of embryos. Authors usually speculate, with no direct confirmation, that differences are indeed associated with abnormal development. The demonstration that certain epigenetic features (e.g., changes in DNA methylation) are common in NT-derived embryos (58, 59, 60, 61) only reinforce the concept that aberrant

phenotypes may be potentially linked to any epigenetically modified gene. Collectively, the wide range variation in embryo phenotypes provides a glimpse about the level of difficulty faced to identify and establish patterns that could be used as markers for developmental abnormalities. Despite many efforts, such markers are not yet available.

Differential patterns of expression can be more often seen at the placental level during development. Arnold et ál. (62) have observed expression of important genes for placental development to be altered in SCNT-derived embryos. Likewise, Humpherys et ál. (63) analyzed global gene expression by microarray analysis of liver and placentas of SCNT-derived newborn mice. Approximately 4% of the analyzed genes showed a significant differential pattern of expression in the placenta, with lesser differences in the liver, with most of these genes being different between the tissue types. The authors concluded that part of the abnormal gene expression may be due to the SCNT procedures, whereas others are due to the donor cell. In cattle, global expression analyses revealed a wide range gene deregulation in the liver of Day 48-56 SCNT-derived fetuses (64). At term, the pattern of gene expression and protein profiles in fetuses and placental tissues have been linked to postnatal survival, with aberrant profiles characterizing deceased newborn and rather normal profiles been associated with surviving adult clones (65, 66, 67). However, the identification of strong correlations between gene profiles at term and postnatal survival is of little value, currently serving more as clues for retrospective understanding of the potential etiology during prenatal development.

Under the current experimental designs and conditions, it is unlikely that studies on gene expression will reveal significant clues for the resolution of the developmental abnormalities caused by *in vitro* embryo manipulations, especially when evaluated at preimplantation stages. For that to be worthy, a change of focus is needed, perhaps for a more holistic approach to the problem, with a departure from the reductionism of the current molecular investigations. In a broad view, the evaluation of same individuals throughout development, checking molecular and physiological patterns and then following phenotypes, are essential. In addition, studies should also evaluate pregnancy losses of natural origin, to understand errors that occur in nature, including natural deviations that do not cause losses or phenotypical abnormalities.

IMPLICATIONS

In view of what have been discussed, we believe that many abnormalities seen after *in vitro* embryo production do occur in nature; *in vitro* embryo manipulations appear to increase their frequency of occurrence. In addition, IVP-derived animals appear to have a narrow physiological window for normality, for which trivial events for most individuals are critical or lethal. The understanding of the physiological mechanisms leading to the LOS is important for the establishment of diagnostic methods that would allow the detection and prevention of the appearance of physiological deviations during pre- and postnatal development. If such processes are identified, distinct patterns could be used as valuable markers for embryos with higher or lower developmental potential. In this view, lower pregnancy rates with higher gestational and postnatal losses represent significant economical losses for a lower prolificacy. Since an extremely high proportion of IVP-derived pregnancies are lost during early pregnancy, embryos from IVF and SCNT technologies are promising study models for embryonic mortality. However, no recognizable and

trustworthy profiles of embryo abnormalities prior to transfer to female recipients that are correlated with the appearance of the LOS are yet available. Also, the early predictability of such spectrum of abnormalities in IVP pregnancies other than the conceptus demise is not yet possible.

Since placental development and function have also been associated with *in vitro* embryo manipulations, changes in placental formation, affecting its stereological structure and tissue micro-architecture, including vasculogenesis and angiogenesis, or causing further changes in placental function and metabolism, may lead to changes in the pattern of fetal development, in a placental-fetal cause-and-effect mechanism. Such mechanisms will likely result in abnormalities for the remaining of the pregnancy, even prevailing throughout the individual's life, as predicted by the Barker's Hypothesis. Prospective future efforts should focus on the pitfalls related to the low overall embryo production efficiency and IVP conditions; on cell differentiation, pluripotency and reprogramming; and on means to improve development after transfer by maximizing pregnancy rates, by minimizing pregnancy losses and excessive prenatal growth, and by improving the adaptation to life *ex utero*. Elucidation of causal factors behind failures is likely to continue increase our understanding of normal events in bovine reproduction and pregnancy that will assist the resolution of problems associated with the LOS and, perhaps, with those of normal occurrence in our herds. All these aspects, if better recognized, will have scientific and economical implications to agriculture and biotechnology.

REFERENCES

1. Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA. Normal development following in vitro fertilization in the cow. Biol Reprod 1982; 27: 147-158.
2. Willadsen SM. Nuclear transplantation in sheep embryos. Nature 1986; 320: 63-65.
3. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385: 810-813.
4. Thibier M. Data Retrieval Committee Annual Report: Transfer of both in vivo derived and in vitro produced embryos in cattle still on the rise and contrasted trends in other species in 2005. Embryo Transfer Newsletter 2006; 24: 12-18.
5. Walker SK, Hartwich KM, Seamark RF. The production of unusually large offspring following embryo manipulation: concepts and challenges. Theriogenology 1996; 45: 111-120.
6. Willadsen SM, Janzen RE, McAlister RJ, Shea BF, Hamilton G, McDermand D. The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. Theriogenology 1991; 35: 161-170.
7. Wilson JM, Williams JD, Bondioli KR, Looney CR, Westhusin ME, McCalla DF. Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating. Anim Reprod Sci 1995; 38: 73-83.
8. Behboodi E, Anderson GB, BonDurant RH, Cargill SL, Kreuscher BR, Medrano JF, Murray JD. Birth of large calves that developed from in vitro-derived bovine embryos. Theriogenology 1995; 44: 227-232.
9. Farin PW, Farin CE. Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development. Biol Reprod 1995; 52: 676-682.
10. Hill JR, Edwards JF, Sawyer N, Blackwell C, Cibelli JB. Placental anomalies in a viable cloned calf. Cloning 2001; 3: 83-88.
11. Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T et ál. Evidence for placental abnormality as the major cause

of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod 2000; 63: 1787-1794.

12. Renard JP, Chastant S, Chesné P, Richard C, Marchal J, Cordonnier N et ál. Lymphoid hypoplasia and somatic cloning. Lancet 1999; 353: 1489-1491.

13. Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. J Reprod Fertil 2000; 120:231-237.

14. Kruip TA, Den Daas JHG. In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. Theriogenology 1997; 47: 43-52.

15. Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R, Niemann H. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. Biol Reprod 2001; 65: 309-317.

16. Farin PW, Crosier AE, Farin CE. Influence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology 2001; 55: 151-170.

17. Yang X, Tian XC, Wakayama T. Nuclear reprogramming of cloned embryos. Nat Genet 2007; 39: 295-302.

18. DeSouza PA, King T, Harkness L, Young LE, Walker SK, Wilmut I. Evaluation of gestational deficiencies in cloned sheep fetuses and placentae. Biol Reprod 2001; 65: 23-30.

19. Bertolini M, Anderson GB. The placenta as a contributor to production of large calves. Theriogenology 2002; 57: 181-187.

20. Garry FB, Adams R, Mccann JP, Odde KG. Postnatal characteristics of calves produced by nuclear transfer cloning. Theriogenology 1996; 45: 141-152.

21. Wells DN, Forsyth JT, McMillan V, Oback B. The health of somatic cell cloned cattle and their offspring. Cloning & Stem Cells 2004; 6: 101-110.

22. Batchelder CA, Hoffert KA, Bertolini M, Moyer AL, Mason JB, Petkov SG et ál. Effect of the nuclear-donor cell lineage, type, and cell donor on development of somatic cell nuclear transfer embryos in cattle. Cloning & Stem Cells 2005; 7: 238-254.

23. Batchelder CA, Bertolini M, Mason JB, Moyer AL, Hoffert KA, Petkov SG et ál. Perinatal physiology in cloned and normal calves: physical and clinical characteristics. Cloning & Stem Cells 2007; 9: 63-82.

24. Batchelder CA, Bertolini M, Mason JB, Moyer AL, Hoffert KA, Petkov SG et ál. Perinatal physiology in cloned and normal calves: hematologic and biochemical profiles. Cloning & Stem Cells 2007; 983-96.

25. Constant F, Guillomot M, Heyman Y, Vignon X, Laigre P, Servely JL et ál. Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. Biol Reprod 2006; 75: 122-130.

26. Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 2006; 65: 178-191.

27. Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol Reprod 2002; 67: 767-775.

28. Bertolini M, Beam SW, Shim H, Bertolini LR, Moyer AL, Famula TR and Anderson GB. Growth, development and gene expression by in vivo- and in vitro-produced Day-7 and Day-16 bovine embryos. Mol Reprod Dev 2002; 63: 318-328.

29. Martin L, Besch-Williford C, Lai L, Cheong HT, Im GS, Park KW et ál. Morphologic and histologic comparisons between in vivo and nuclear transfer derived porcine embryos. Mol Reprod Dev 2007; 74: 952-960.

30. Bertolini M, Mason JB, Beam SW, Carneiro GF, Sween ML, Moyer AL et ál. Morphology and morphometry of in vivo- and in vitro-produced bovine concepti from early pregnancy to term and association with high birth weights. Theriogenology 2002; 58: 973-994.

31. Chavatte-Palmer P, De Sousa N, Laigre P, Camous S, Ponter AA, Beckers JF, Heyman Y. Ultrasound fetal measurements and pregnancy associated glycoprotein secretion in early pregnancy in cattle recipients carrying somatic clones. Theriogenology 2006; 66: 829-840.

32. Bertolini M, Moyer AL, Mason JB, Batchelder CA, Hoffert KA, Bertolini LR et ál. Evidence of increased substrate availability to in vitro-derived bovine foetuses and association with accelerated conceptus growth. Reproduction 2004; 128: 341-354.

33. Bertolini M, Wallace CR, Anderson GB. Expression profile and protein levels of placental products as indirect measures of placental function in in vitro-derived bovine pregnancies. Reproduction 2006; 131: 163-173.

34. Hill JR, Chavatte-Palmer P. Pregnancy and neonatal care of cloned animals. In: Cibelli J, Lanza RP, Campbell KHS, West MD (eds.). Principles of cloning. Amsterdam: Academic Press; 2002. p. 247-266.

35. Heyman Y, Chavatte-Palmer P, LeBourhis D, Camous S, Vignon X, Renard JP. Frequency and occurrence of late-gestation losses from cattle cloned embryos. Biol Reprod 2002; 66: 6-13.

36. Wells DN, Misica PM, Tervit HR. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biol Reprod 1999; 60: 996-1005.

37. Hill JR, Schlafer DH, Fisher PJ, Davies CJ. Abnormal expression of trophoblast major histocompatibility complex class I antigens in cloned bovine pregnancies is associated with a pronounced endometrial lymphocytic response. Biol Reprod 2002; 67: 55-63.

38. Lee RS, Peterson AJ, Donnison MJ, Ravelich S, Ledgard AM, Li N et ál. Cloned cattle fetuses with the same nuclear genetics are more variable than contemporary halfsiblings resulting from artificial insemination and exhibit fetal and placental growth deregulation even in the first trimester. Biol Reprod 2004; 70: 1-11.

39. Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA et ál. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 1995; 43: 141-152.

40. Li N, Wells DN, Peterson AJ, Lee RS. Perturbations in the biochemical composition of fetal fluids are apparent in surviving bovine somatic cell nuclear transfer pregnancies in the first half of gestation. Biol Reprod 2005; 73: 139-148.

41. Miles JR, Farin CE, Rodriguez KF, Alexander JE, Farin PW. Effects of embryo culture on angiogenesis and morphometry of bovine placentas during early gestation. Biol Reprod 2005; 73: 663-671.

42. Fletcher CJ, Roberts CT, Hartwich KM, Walker SK, McMillen IC. Somatic cell nuclear transfer in the sheep induces placental defects that likely precede fetal demise. Reproduction 2007; 133: 243-255.

43. Palmieri C, Loi P, Reynolds LP, Ptak G, Della Salda L. Placental abnormalities in ovine somatic cell clones at term: a light and electron microscopic investigation. Placenta 2007; 28: 577-584.

44. Miglino MA, Pereira FTV, Visintin JA, Garcia JM, Meirelles FV, Rumpf R et ál. Placentation in cloned cattle: structure and microvascular architecture. Theriogenology 2007; 68: 604-617.

45. Matsuzaki M, Shiga K. Endocrine characteristics of cloned calves. Cloning & Stem Cells 2002; 4: 261-267.

46. Chavatte-Palmer P, Remy D, Cordonnier N, Richard C, Issenman H, Laigre P et ál. Health status of cloned cattle at different ages. Cloning & Stem Cells 2004; 6: 94-100.

47. Rudenko L, Matheson JC, Adams AL, Dubbin ES, Greenlees KJ. Links Food consumption risks associated with animal clones: what should be investigated? Cloning & Stem Cells 2004; 6: 79-93.

48. Loi P, Clinton M, Vackova I, Fulka J Jr, Feil R, Palmieri C et ál. Placental abnormalities associated with post-natal mortality in sheep

somatic cell clones. Theriogenology 2006; 65: 1110-1121.

49. Mcmillen CI, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev 2005; 85: 571-633.

50. Bertram CA, Hanson MA. Animal models and programming of the metabolic syndrome. Br Med Bull 2001; 60: 103-121.

51. Barker DJP. Fetal programming and public health. In: O'Brien PMS, Wheeler T, Barker DJP (Ed.). Fetal programming: influences on development and disease in later life. London: RCOG Press; 1999. p. 3-11.

52. Hall VJ, Ruddock NT, French AJ. Expression profiling of genes crucial for placental and preimplantation development in bovine in vivo, in vitro, and nuclear transfer blastocysts. Mol Reprod Dev 2005; 72: 16-24.

53. Inoue F, Matsuda J, Ohkoshi K, Furusawa T, Takahashi S, Sasada H et ál. Differences in gene expression patterns between somatic cell nuclear transfer embryos constructed with either rabbit granulosa cells or their derivatives. Anim Reprod Sci 2006; 93: 76-87.

54. Li X, Amarnath D, Kato Y, Tsunoda Y. Analysis of development-related gene expression in cloned bovine blastocysts with different developmental potential. Cloning & Stem Cells 2006; 8: 41-50.

55. Moreira PN, Fernández-González R, Ramírez MA, Pérez-Crespo M, Rizos D, Pintado B, Gutiérrez-Adan A. Differential effects of culture and nuclear transfer on relative transcript levels of genes with key roles during preimplantation. Zygote 2006; 14: 81-87.

56. Beyhan Z, Forsberg EJ, Eilertsen KJ, Kent-First M, First NL. Gene expression in bovine nuclear transfer embryos in relation to donor cell efficiency in producing live offspring. Mol Reprod Dev 2007; 74: 18-27.

57. Smith C, Berg D, Beaumont S, Standley NT, Wells DN, Pfeffer PL. Simultaneous gene quantitation of multiple genes in individual bovine nuclear transfer blastocysts. Reproduction 2007; 133: 231-242.

58. Bourc'his D, Le Bourhis D, Patin D, Niveleau A, Comizzoli P, Renard JP, Viegas-Pequignot E. Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. Curr Biol 2001; 11: 1542-1546.

59. Kang YK, Koo DB, Park JS, Choi YH, Chung AS, Lee KK, Han YM. Aberrant methylation of donor genome in cloned bovine embryos. Nature Genet 2001; 28: 173-177.

60. Beaujean N, Taylor J, Gardner J, Wilmut I, Meehan R, Young L. Effect of limited DNA methylation reprogramming in the normal sheep embryo on somatic cell nuclear transfer. Biol Reprod 2004; 71: 185-193.

61. Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nat Genet 2007; 39: 295-302.

62. Arnold DR, Bordignon V, Lefebvre R, Murphy BD, Smith LC. Somatic cell nuclear transfer alters peri-implantation trophoblast differentiation in bovine embryos. Reproduction 2006; 132: 279-290.

63. Humpherys D, Eggan K, Akutsu H, Friedman A, Hochedlinger K, Yanagimachi R et ál. Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. Proc Natl Acad Sci USA 2002; 99: 12889-12894.

64. Herath CB, Ishiwata H, Shiojima S, Kadowaki T, Katsuma S, Ushizawa K et ál. Developmental aberrations of liver gene expression in bovine fetuses derived from somatic cell nuclear transplantation. Cloning & Stem Cells 2006; 8: 79-95.

65. Kim HR, Kang JK, Yoon JT, Seong HH, Jung JK, Lee HM et ál. Protein profiles of bovine placenta derived from somatic cell nuclear transfer. Proteomics 2005; 5: 4264-4273.

66. Li S, Li Y, Du W, Zhang L, Yu S, Dai Y et ál. Aberrant gene expression in organs of bovine clones that die within two days after birth. Biol Reprod 2005; 72: 258-265.

67. Yang L, Chavatte-Palmer P, Kubota C, O'Neill M, Hoagland T, Renard JP et ál. Expression of imprinted genes is aberrant in deceased newborn cloned calves and relatively normal in surviving adult clones. Mol Reprod Dev 2005; 71: 431-438.

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