Embryo technology: implications for fertility in cattle

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Summary

During the past thirty years, basic and experimental studies on classical (superovulation; non-surgical recovery and transfer of cattle embryos) and advanced embryo technologies (*in vitro* embryo production; cloning by somatic cell nuclear transfer) have generated structural and functional information on oocyte development and quality, fertilisation and conceptus development. This information has provided new insight, not only into these technologies *per se* but also into the factors contributing to fertility in cattle. It is now known that the peripheral and follicular endocrine profiles have a profound influence on the subsequent developmental competence of the embryo. It is also well established that manipulation of the oocytes or embryos may adversely affect embryonic and foetal development, leading to the so-called 'large offspring syndrome'. Information from such studies has alerted scientists to the importance of epigenetics in cattle reproduction.

Keywords

Assisted reproductive technology – Bovine – Cloning – Embryo technology – Embryo transfer – Epigenetics – Fertility – Follicle – Follicular wave pattern – In vitro production – Oocyte – Ovulation – Reproduction – Superovulation.

Introduction

Techniques for the non-surgical recovery of six-to-seven-day-old cattle embryos from animals that had been superovulated with gonadotrophin serum from pregnant mares were first conducted in the mid-1970s. These techniques provided a promising new tool for cattle breeding. Embryo transfer was soon added to the more classical technique of artificial insemination (AI), which was already in routine use. This development introduced an entirely new and exciting era of research in reproductive biotechnology in cattle. Superovulation, non-surgical embryo recovery from donor animals and non-surgical embryo transfer to oestrus-synchronised recipients were rapidly succeeded by more advanced embryo-technological methods, such as:

– cryopreservation
– bisection of embryos
– sexing of embryos
– *in vitro* production
– cloning of embryos
– transgenic animal production
– cloning by somatic cell nuclear transfer.

Perfecting each of these embryo technologies required intensive basic and applied research, which not only yielded important information for that specific technique but also generated scientific data that could be used in other contexts.

Initially, much attention was placed on the recovered embryos and the various ways in which they could be handled. However, subsequent steps in the process, leading to live births from these embryos, have proved to be equally important areas for study. For instance, the extent of conceptus loss and its often hidden causes is now far better understood. This is principally due to deeper insights into the structure and function of the follicle, oocyte and early development of the embryo (maternalzygotic transition, apoptosis and chromosomal aberrations) and a clearer understanding of the processes of pregnancy and even parturition.

Embryo technologies have led to a more profound understanding of the entire continuum of conception, pregnancy and birth, thus teaching researchers a great deal about fertility. In this very brief paper, the authors address the question of what embryo technology and its related disciplines have demonstrated in the fields of normal and abnormal fertility in cattle.

The follicle

A very high number (> 100,000) of resting primordial follicles are present in the ovary of the bovine female at birth. These follicles are formed during foetal life and will continuously be recruited to either ovulation or natural demise (atresia) during the lifetime of the cow. Most will undergo atresia. The growth period of the follicle from the early stages until ovulation is estimated to be approximately three months (11).

Through daily rectal ultrasonography and subsequent retrospective analysis of the recordings, it has been well established that more than 95% of all oestrous cycles consist of two or three follicular waves (1, 10). The pattern is fairly repeatable within a given animal (24) but is apparently independent of age and species (10).

The pattern of follicular wave growth and selection of the dominant follicles of each wave have been described in detail in several reviews and will not be addressed here (1, 10, 12). The hormonal and molecular mechanisms regulating growth, deviation and the final selection of the dominant follicle are very complex and include endocrine and paracrine processes. However, it is clear that circulating concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play a major role, in conjunction with inhibin, activin, follistatin and several growth factors (12, 32, 41). The ever-increasing knowledge about follicular growth, deviation and atresia has given researchers a better understanding of variations in the length of cattle oestrous cycles (the three-wave pattern lasts between one and two days longer than the two-wave pattern). It has also helped to explain why a certain proportion of heifers and cows are wrongly inseminated from around day 10 to day 12. These days coincide with the existence of the oestrogen-producing dominant follicle of the first follicular wave, giving rise to oestrus-like symptoms. Although the oocyte contained in this follicle is competent and may be fertilised and undergo normal embryonic development, ovulation of the follicle is extremely rare in cattle because of the high progesterone level and lack of an LH peak. Removed from its follicle, such a mature cumulus oocyte complex (COC) may be fertilised *in vitro*, and reports on the birth of twins approximately 10 to 14 days apart may well be a consequence of ovulation and fertilisation of the oocyte from both the first and second follicular waves. It should be emphasised that a better understanding of the very finely tuned regulation of follicular deviation and ovulation may explain why even subtle changes in feeding, stress, etc., may disrupt these processes and result in, for example, cystic ovaries (46).

Information obtained about the follicular wave pattern over the past few years has also been useful in the context of embryo technology. First, it has enabled researchers to achieve better superovulation protocols and even to manipulate the follicular wave (3). Secondly, it explains why it is better to recover oocytes through ovum collection or 'ovum pick-up' (OPU) twice weekly rather than once a week (34). Twice weekly OPU sessions suppress the normal follicular wave pattern and thus result in a more consistent number and even a more uniform population of follicles with their enclosed COC.

The oocyte

The oocytes present in the ovaries of the newborn heifer calf are maintained in the pro-phase of the first meiotic division. They remain in this state until final nuclear maturation begins, approximately 24 hours before ovulation. However, to be developmentally competent and ready for ovulation, the oocyte must undergo a long series of specific changes, encompassing oocyte growth, capacitation and the final pre-ovulatory maturation.

The growth phase is by far the longest period and is estimated to be approximately six months. During this period, the diameter of the oocyte increases from around 30 µm in the primordial follicle to more than 130 µm in the tertiary follicle. Very important structural and molecular changes occur within the ooplasm during this growth period, which provide the oocyte with the required developmental competences for normal embryonic and foetal development (11, 23, 30, 38).

In the primary and secondary follicles, the oocyte builds up a reserve of ribonucleic acid, proteins, lipids and carbohydrates for later use, that is, when the embryo becomes transcriptionally active at approximately the eight-cell stage. Accompanying, and obviously dependent upon, these reserves, certain organelles appear, namely: the Golgi complex, the endoplasmatic reticulum, mitochondriae, vesicles and lipid droplets.

In the secondary follicle, the *zona pellucida* is formed and communication between the oocyte and the surrounding follicular environment is assured by cumulus cell projections through the *zona*. At this stage the cortical granules are also formed.

Most of the processes that take place in this growth phase are aimed at consolidating and fine-tuning the ooplasmic competence, whereas nuclear changes occur at a later stage of development.

During the growth phase, oocytes may be exposed to a number of adverse conditions which may directly compromise oocyte quality and later embryonic development (5), thus having a significant effect on post-partum fertility. Such conditions include:

– disrupting compounds (for example, hormones)
– adverse feeding regimens
– heat stress
– toxins
– infections.

However, there may also be significant and long-lasting effects on pregnancy, parturition and post-natal development when normal oocyte development is only slightly disrupted.

The changes that occur within the oocyte, from the time its follicle is selected for dominance until the pre-ovulatory LH surge, are known as 'oocyte capacitation' (23). The processes which happen during this period are specifically aimed at preparing the oocyte for imminent ovulation and fertilisation and include, as follows:

– the peripheral localisation of organelles with fewer Golgi complexes
– the production of more lipid droplets
– the peripheral migration of cortical granules to positions under the plasma membrane.

In addition, the nuclear membrane shows signs of initial breakdown and the nucleolus undergoes specific changes.

The final phase, the pre-ovulatory period, is initiated by the pre-ovulatory LH surge and ends with ovulation approximately 24 hours later (6). The ultrastructural changes which occur during this period have been described in detail by the authors *et al.* in Denmark (23). These changes encompass, for example:

– the loss of contact between the cumulus projections and the oocyte
– the peripheral distribution of the cortical granules
– an increase in lipid and protein stores
– the resumption of meiosis.

Immediately before ovulation, the oocyte is at metaphase II in the second meiotic division.

The interaction between follicular environment and oocyte

It has become increasingly evident, during the era of superovulation and embryo transfer (14, 16), as a result of the experience gained from both human and animal *in vitro* fertilisation studies (15), that the follicular micro-environment may affect oocyte quality, its ability to be fertilised and subsequent embryonic development. Conversely, the follicular endocrine dynamic is profoundly affected by the endocrine balance and hormonal profiles of the donor (6, 15, 16). The subtle and finely tuned structural, molecular and endocrine changes are well synchronised and in homeostatic balance in the 'normal' heifer or cow. This synchronised balance can obviously be easily disrupted by factors that interfere with normal endocrine regulation, the follicular micro-environment and hence the final maturation of the oocyte and (delayed) ovulation. The consequences are both immediate (lack of fertilisation and of the ability of the embryo to undergo early development to the blastocyst stage) and long-lasting, as reflected by the occurrence of large offspring syndrome.

Newer experiments from Utrecht have clearly demonstrated the differences between the quality of *in vivo*-matured oocytes and those matured *in vitro* (9). These changes are also reflected in differences in the transcriptional patterns of the resulting embryos produced *in vivo*, compared to those produced *in vitro* (33, 47). Whether the follicle or the oocyte is the determining element in achieving final competence has yet to be discovered but newer research seems to indicate that the oocyte plays a very important role in this process (11).

Ovulation and the oviduct

Recent experiments have clearly substantiated that, as the follicle approaches ovulation, its temperature decreases. In fact, the follicle becomes cooler than the surrounding stroma by approximately one to one-and-a-half degrees Celsius (17, 22). The precise mechanism is not yet known but, according to Hunter (21), is probably due to chemical reactions in the follicle combined with some kind of counter-current system in both the follicle and the ovary. Since the temperature is lower in the follicles, one might argue that it would be advantageous to perform the final *in vitro* oocyte maturation at a lower temperature than the normally used 38.5°C to 39.0°C. This was tested some years ago but the study provided no specific conclusions (37).

The normal ovulation process has recently been described in great detail (21). Among the changes occurring are the following:

– reduced permeability of the vessels of the theca layer
– increased viscosity of the follicular fluid
– increased volume of the follicular fluid
– increased intrafollicular pressure.

The duration of ovulation is between one and three minutes, approximately, and occurs as oozing, where the oocyte with its surrounding cumulus cells is caught by the fimbriae in the oviduct (21).

It is very clear, from studies in superovulated cattle, that the oocyte must leave the follicle at a very specific and predetermined time interval following the LH surge. Delayed or disrupted ovulation will inevitably lead to oocytes of inferior quality (14). This observation has been confirmed by studies in which ovulation was disrupted by preventing or delaying the LH surge (13). In unstimulated animals, a similar situation may well arise in conjunction with stress (high milk yield, improper feeding), which is known to interfere with the hormonal regulation of ovulation and thus produce 'overmatured' oocytes, most of which cannot be fertilised. However, if fertilised, the zygotes are of inferior quality and lead to an increased incidence of early embryonic mortality. Collectively, this will result in the reduced reproductive capacity of a herd.

The functioning of the oviduct at ovulation and throughout early embryonic development has been described in detail (20, 21), and is apparently regulated by the relative concentrations of progesterone and estradiol-17, which reach the fallopian tube through the counter-current system of the oviductal vessels. The hormonal profiles of superovulated animals before, during and after ovulation may be disrupted (6), leading to an adverse environment in the oviduct. This may ultimately result in improper sperm transport and reduced or abolished fertilisation, evidenced by a reduced number of supernumerary spermatozoa in the *zona pellucida* (36). Oedema at the site of the utero-tubal junction and enhanced or reduced oviductal motility are other factors leading to lack of fertilisation or improper embryonic development. Again, it should be stressed that similar conditions may occur in animals which have not undergone superovulation. It is reasonable to extrapolate from the data gathered on superovulated animals to non-stimulated (normal) animals.

Applying the lessons of embryo technology to fertility

The processes described above, namely, follicular development; oocyte growth, capacitation and maturation; ovulation, fertilisation, conceptus development and parturition, are under increasing pressure, leading to reduced fertility in dairy herds (28). The overall calving rate may be as low as 33% on day 28 following one AI (27). Many factors, such as nutrition (4), genetics (35) and yield (5), individually or in combination, are contributing to this trend. In addition, an increasing incidence of calf mortality is producing an even more drastic trend for dairy cattle production: there are, quite simply, not enough replacement heifers.

Superovulation studies that include measurements of peripheral as well as follicular endocrine parameters clearly indicate that oocyte quality may be reduced in superstimulated animals. However, when the embryos have reached the blastocyst stage on day 7, their qualities are apparently similar to those of non-recovered embryos, since the pregnancy rates and conceptus loss rates following transfer to recipient animals are similar to those found in AI data (7). Abnormal endocrine patterns following superstimulation might also contribute to an adverse environment in the oviduct and thus reduced fertility. Again, the crucial periods are those of fertilisation and early embryo development. The superovulatory era also led to the categorisation of embryo quality and there are now well-defined standards, set by the International Embryo Transfer Society. This, in itself, is an achievement.

The era of *in vitro* fertilisation has provided important new information about the fertilisation process, early embryo development and embryo quality. However, *in vitro* embryos are also fundamentally different from their *in vivo* counterparts in terms of, for example, morphology (8, 29), gene expression patterns (26, 33, 47) and chromosomal abnormalities (39, 40). The *in vitro* era has also made it possible to obtain a much more accurate understanding of oocyte quality and the factors which affect this (31). Through detailed morphological and molecular studies, it has become possible to predict the quality of a given oocyte and thus to estimate whether it will produce a normal embryo and conceptus development. An even more important gain is the potential to quantify factors that might lead to poorer oocyte quality, such as an adverse endocrine environment, heat stress, etc., because this may explain why *in vivo* processes often deteriorate.

Some of the most important information which has been gained from the *in vitro* production era (including the production of embryos by embryo and somatic cell cloning) is the so-called 'large offspring syndrome', which was first reported in 1991 (45), and later addressed in a more comprehensive review (25). It is now accepted that the transfer of *in vitro*-produced and cloned embryos frequently results in the following:

– a high rate of conceptus loss
– abnormal foetal and placental growth and development (hydrallantois)

– larger and weaker calves
– a weak labour (2, 18, 19, 42, 43, 44).

It has been well established that disruption of the expression pattern of certain genes which are important in development, including imprinted genes, is involved in this syndrome (48).

All these factors can adversely affect reproductive capacity. However, there is also a new element to be taken into account: epigenetics. There is no doubt that epigenetics is profoundly involved in embryo and foetal losses in cattle, not only under artificial conditions but also under normal ones. One could say that *in vitro* embryo production and, in particular, cloning by somatic cell nuclear transfer exaggerate the problems and thus make it easier to deduce the causes of embryo, conceptus and neonatal mortality. In studying these magnified phenomena, researchers have learned that similar mechanisms occur under normal conditions. The result is a much better understanding of so-called 'normal' embryo and conceptus loss.

Conclusion

Over the past twenty-five years, superovulation and embryo technology have become a crucial part of cattle breeding in most parts of the world. In addition, the underlying research has clarified various features of endocrinology, culture conditions and manipulations which may negatively affect oocyte and embryo quality, and thus help to explain reduced fertility in cattle.

The science and practice of artificial embryo production (*in vitro* production and cloning) have given researchers valuable insight into the importance of the early embryonic period for later foetal and neonatal development. How epigenetics may be influenced, how normal imprinting patterns may be disrupted and to what extent these processes contribute to abnormal development are issues that remain to be clarified. Through studying in greater detail the aberrant features of artificially produced embryos, researchers may find that the very same mechanisms lie behind so-called 'normal' foetal and neonatal losses. In this way, progress may be made towards practical solutions.

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Technologies embryonnaires : implications pour la fertilité chez les bovins

T. Greve & H. Callesen

Résumé
Au cours de ces 30 dernières années, les études théoriques et expérimentales sur les techniques embryonnaires classiques (superovulation, prélèvement non chirurgical et transfert d'embryons de bovins) et sur les technologies avancées (production d'embryons *in vitro*, clonage par transfert nucléaire somatique) ont donné lieu à des informations structurales et fonctionnelles sur le développement et la qualité des ovocytes, la fertilisation et le développement embryonnaire. Ces informations ont ouvert de nouvelles perspectives, non seulement sur ces technologies en tant que telles mais également sur les facteurs contribuant à la fertilité chez des bovins. On sait à présent que les profils endocriniens périphériques et folliculaires ont une influence profonde sur la capacité de développement ultérieure de l'embryon. Il est également bien

établi que la manipulation des ovocytes ou des embryons peut avoir un effet défavorable sur le développement embryonnaire et fœtal et donner lieu au « syndrome du gros veau ». Les informations issues de ces études ont ouvert les yeux des scientifiques sur l'importance des facteurs épigénétiques pour la reproduction chez les bovins.

Mots-clés
Bovin – Clonage – Facteur épigénétique – Fertilité – Follicule – Ovocyte – Ovulation – Production in vitro – Reproduction – Superovulation – Technique de reproduction assistée – Technologie embryonnaire – Transfert d'embryons – Vague folliculaire.

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Efectos sobre la fertilidad bovina de las técnicas de manipulación de embriones

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Resumen
En los últimos treinta años, los estudios básicos y experimentales sobre las técnicas de manipulación de embriones, ya sean convencionales (superovulación, extracción y transferencia no quirúrgicas de embriones bovinos) o avanzadas (obtención *in vitro* de embriones, clonación por transferencia de núcleos de células somáticas), han ido generando información sobre los aspectos estructurales y funcionales del desarrollo de los ovocitos, su calidad, la fertilización o el desarrollo del conceptus, información que ha arrojado nueva luz no sólo sobre esas técnicas en sí mismas sino también sobre los factores que contribuyen a la fertilidad del ganado. Se sabe ahora que el perfil endocrino, tanto periférico como folicular, influye sobremanera en la posterior capacidad de desarrollo del embrión. También está demostrado que la manipulación de los ovocitos o embriones puede influir negativamente en el desarrollo embrionario o fetal y generar lo que da en llamarse el 'síndrome de la cría grande'. La información obtenida con esos estudios ha revelado a los científicos la importancia de la epigenética en la reproducción del ganado.

Palabras clave
Bovino – Clonación – Epigenética – Fertilidad – Folículo – Onda folicular – Ovocito – Ovulación – Producción in vitro – Reproducción – Superovulación – Técnica de manipulación de embriones – Técnica de reproducción asistida – Transferencia de embriones.

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References

1. Adams G.P. (1999). – Comparative patterns of follicle development and selection in ruminants. *J. Reprod. Fertil.*, **54** (Suppl.), 17-32.

2. Bertolini M., Mason J.B., Beam S.W., Carneiro G.F., Sween M.L., Kominek D.J., Moyer A.L., Famula T.R., Sainz R.D. & Anderson G.B. (2002). – Morphology and morphometry of *in vivo*- and *in vitro*-produced bovine concepti from early pregnancy to term and association with high birth weights. *Theriogenology*, **58** (5), 973-994.

3. Bo G.A., Baruselli P.S., Moreno D., Cutaia L., Caccia M., Tribulo R., Tribulo H. & Mapletoft R.J. (2002). – The control of follicular wave development for self-appointed embryo transfer programs in cattle. *Theriogenology*, **57** (1), 53-72.

4. Boland M.P., Lonergan P. & O'Callaghan D. (2001). – Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. *Theriogenology*, **55** (6), 1323-1340.

5. Butler W.R. (2003). – Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. *Livest. Prod. Sci.*, **83** (2/3), 211-218.

6. Callesen H., Greve T. & Hyttel P. (1986). – Preovulatory endocrinology and oocyte maturation in superovulated cattle. *Theriogenology*, **25** (1), 71-86.

7. Callesen H., Liboriussen T. & Greve T. (1996). – Practical aspects of multiple ovulation – embryo transfer in cattle. *Anim. Reprod. Sci.*, **42** (1-4), 215-226.

8. Crosier A.E., Farin P.W., Dykstra M.J., Alexander J.E. & Farin C.E. (2001). – Ultrastructural morphometry of bovine blastocysts produced *in vivo* or *in vitro*. *Biol. Reprod.*, **64** (5), 1375-1385.

9. Dieleman S.J., Hendriksen P.J.M., Viuff D., Thomsen P.D., Hyttel P., Knijn H.M., Wrenzycki C., Kruip T.A.M., Niemann H., Gadella B.M., Bevers M.M. & Vos P.L.A.M. (2002). – Effects of *in vivo* prematuration and *in vivo* final maturation on developmental capacity and quality of pre-implantation embryos. *Theriogenology*, **57** (1), 5-20.

10. Evans A.C.O. (2003). – Characteristics of ovarian follicle development in domestic animals. *Reprod. dom. Anim.*, **38** (4), 240-246.

11. Fair T. (2003). – Follicular oocyte growth and acquisition of developmental competence. *Anim. Reprod. Sci.*, **78** (3-4), 203-216.

12. Ginther O.J. (2000). – Selection of the dominant follicle in cattle and horses. *Anim. Reprod. Sci.*, **60-61**, 61-79.

13. Goff A.K., Greve T., Bousquet D. & King W.A. (1986). – Progesterone and luteinizing hormone profiles in heifers used as oocyte donors. *Theriogenology*, **26** (5), 577-586.

14. Greve T., Bousquet D., King W.A. & Betteridge K.J. (1984). – *In vitro* fertilization and cleavage of *in vivo* matured bovine oocytes. *Theriogenology*, **22** (2), 151-165.

15. Greve T., Callesen H. & Hyttel P. (1989). – Follicular correlates with *in-vitro* fertilization in cattle. *J. Reprod. Fertil.*, **38** (Suppl.), 117-126.

16. Greve T., Callesen H., Hyttel P., Høier R. & Assey R. (1995). – The effects of exogenous gonadotropins on oocyte and embryo quality in cattle. *Theriogenology*, **43** (1), 41-50.

17. Greve T., Grøndahl C., Schmidt M., Hunter R.H.F. & Avery B. (1996). – Bovine preovulatory follicular temperature: implications for *in vitro* production of embryos. *Arch. Tiersucht*, **39** (Spec. issue), 7-14.

18. Hasler J.F. (1998). – The current status of oocyte recovery, *in vitro* embryo production, and embryo transfer in domestic animals, with an emphasis on the bovine. *J. anim. Sci.*, **76** (Suppl. 3), 52-74.

19. Heyman Y., Chavatte-Palmer P., LeBourhis D., Camous S., Vignon X. & Renard J.P. (2002). – Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biol. Reprod.*, **66** (1), 6-13.

20. Hunter R.H.F. (1988). – The fallopian tubes: their role in fertility and infertility. Springer-Verlag, New York, Berlin and Heidelberg, 191 pp.

21. Hunter R.H.F. (2003). – Physiology of the Graafian follicle and ovulation. Cambridge University Press, New York, 397 pp.

22. Hunter R.H.F., Bøgh I.B., Einer-Jensen N., Müller S. & Greve T. (2000). – Pre-ovulatory Graafian follicles are cooler than neighbouring stroma in pig ovaries. *Hum. Reprod.*, **15** (2), 273-283.

23. Hyttel P., Fair T., Callesen H. & Greve T. (1997). – Oocyte growth, capacitation and final maturation in cattle. *Theriogenology*, **47** (1), 23-32.

24. Ireland J.J. & Burns D.S. (2003). – Numbers of antral follicles that grow during different follicular waves in individual cattle are highly repeatable. *Biol. Reprod.*, **68** (Suppl.), 327.

25. Kruip T.A.M. & den Daas J.H.G. (1997). – *In vitro* produced and cloned embryos: effects on pregnancy, parturition and offspring. *Theriogenology*, **47** (1), 43-52.

26. Lonergan P., Rizos D., Gutierrez-Adan A., Fair T. & Boland M.P. (2003). – Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns. *Reprod. dom. Anim.*, **38** (4), 259-267.

27. Lopes A.S., Butler S.T., Gilbert R.O. & Butler W.R. (2004). – The follicle's influence on embryonic/fetal mortality in cattle. *In* Proc. 20th Scientific Meeting of European Embryo Transfer Association (AETE), 10-11 September, Lyons, France. AETE, Lyons, 146 pp.

28. Lucy M.C. (2003). – Mechanisms linking nutrition and reproduction in postpartum cows. *Reproduction*, **61** (Suppl.), 415-427.

29. Maddox-Hyttell P., Gjørret J.O., Vajta G., Alexopoulos N.I., Lewis I., Trounson A., Viuff D., Laurincik J., Müller M., Tveden-Nyborg P. & Thomsen P.D. (2003). – Morphological assessment of pre-implantation embryo quality in cattle. *Reproduction*, **61** (Suppl.), 103-116.

30. Mermillod P., Oussaid B. & Cognié Y. (1999). – Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *J. Reprod. Fertil.*, **54** (Suppl.), 449-460.

31. Merton J.S., de Roos A.P.W., Mullaart E., de Ruigh L., Kaal L., Vos P.L.A.M. & Dieleman S.J. (2003). – Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology*, **59** (2), 651-674.

32. Mihm M. & Bleach E.C.L. (2003). – Endocrine regulation of ovarian antral follicle development in cattle. *Anim. Reprod. Sci.*, **78** (3-4), 217-237.

33. Niemann H., Wrenzycki C., Lucas-Hahn A., Brambrink T., Kues W.A. & Carnwath J.W. (2002). – Gene expression patterns in bovine *in vitro*-produced and nuclear transfer-derived embryos and their implications for early development. *Cloning Stem Cells*, **4** (1), 29-38.

34. Petyim S. (2002). – Ovum pick-up in dairy heifers. PhD thesis. Swedish University of Agricultural Sciences, Uppsala, Sweden, 44 pp.

35. Royal M.D., Flint A.P.F. & Woolliams J.A. (2002). – Genetic and phenotypic relationships among endocrine and traditional fertility traits and production traits in Holstein-Friesian dairy cows. *J. Dairy Sci.*, **85** (4), 958-967.

36. Saacke R.G., Nadir S. & Nebel R.L. (1994). – Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. *Theriogenology*, **41** (1), 45-50.

37. Shi D.S., Avery B. & Greve T. (1998). – Effects of temperature gradients on *in vitro* maturation of bovine oocytes. *Theriogenology*, **50** (4), 667-674.

38. Sirard M.-A., Dufort I., Coenen K., Tremblay K., Massicotte L. & Robert C. (2003). – The use of genomics and proteomics to understand oocyte and early embryo functions in farm animals. *Reproduction*, **61** (Suppl.), 117-129.

39. Viuff D., Hendriksen P.J.M., Vos P.L.A.M., Dieleman S.J., Bibby B.M., Greve T., Hyttel P. & Thomsen P.D. (2001). – Chromosomal abnormalities and developmental kinetics in *in vivo*-developed cattle embryos at days 2 to 5 after ovulation. *Biol. Reprod.*, **65** (1), 204-208.

40. Viuff D., Palsgaard A., Rickords L., Lawson L.G., Greve T., Schmidt M., Avery B., Hyttel P. & Thomsen P.D. (2002). – Bovine embryos contain a higher proportion of polyploid cells in the trophectoderm than in the embryonic disc. *Molec. Reprod. Dev.*, **62** (4), 483-488.

42. Wagtendonk-de Leeuw A.M. van, Aerts B.J.G. & den Daas J.H.G. (1998). – Abnormal offspring following *in vitro* production of bovine preimplantation embryos: a field study. *Theriogenology*, **49** (5), 883-894.

43. Wagtendonk-de Leeuw A.M. van, Mullaart E., de Roos A.P.W., Merton J.S., den Daas J.H.G., Kemp B. & de Ruigh L. (2000). – Effects of different reproduction techniques: AI, MOET or IVP, on health and welfare of bovine offspring. *Theriogenology*, **53** (2), 575-597.

41. Webb R., Nicholas B., Gong J.G., Campbell B.K., Gutierrez C.G., Garverick H.A. & Armstrong D.G. (2003). – Mechanisms regulating follicular development and selection of the dominant follicle. *Reproduction*, **61** (Suppl.), 71-90.

44. Wells D.N., Laible G., Tucker F.C., Miller A.L., Oliver J.E., Xiang T., Forsyth J.T., Berg M.C., Cockrem K., L'Huillier P.J., Tervit H.R. & Oback B. (2003). – Coordination between donor cell type and cell cycle stage improves nuclear cloning efficiency in cattle. *Theriogenology*, **59** (1), 45-59.

45. Willadsen S.M., Janzen R.E., McAlister R.J., Shea B.F., Hamilton G. & McDermand D. (1991). – The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. *Theriogenology*, **35** (1), 161-170.

46. Wiltbank M.C., Gümen A. & Sartori R. (2002). – Physiological classification of anovulatory conditions in cattle. *Theriogenology*, **57** (1), 21-52.

47. Wrenzycki C., Lucas-Hahn A., Herrmann D., Lemme E., Korsawe K. & Niemann H. (2002). – *In vitro* production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. *Biol. Reprod.*, **66** (1), 127-134.

48. Young L.E. & Fairburn H.R. (2000). – Improving the safety of embryo technologies: possible role of genomic imprinting. *Theriogenology*, **53** (2), 627-648.