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Application of embryo transfer using *in vitro* produced embryos: intrinsic factors affecting efficiency

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Abstract

Embryo transfer remains a viable approach to increase propagation of offspring from high genetic merit females. Although it is now over 60 years since the report of the birth of the first calf from embryo transfer, utilisation of embryo transfer technology worldwide is not widespread. Limitations of conventional procedures for superovulation and embryo transfer are not limited to but include variability in response to superovulation, the labour intensive nature of superovulation procedures, time required between collections and cost of technology. Recently, harvest of ova and transfer of *in vitro* produced embryos has received more attention as a potential alternative to conventional superovulation and subsequent embryo transfer. Aspiration of follicular ova and *in vitro* embryo production offers potential advantages in reducing loss of female germplasm occurring through the natural process of ovarian follicular atresia, can increase yield of embryos from elite donor cows beyond that possible with superovulation, and provides a means of salvaging genetic material from valuable animals at slaughter or those culled for disease control or other reasons. Recent evidence indicates poor ovum quality is a major factor limiting *in vitro* embryo production and discovery of a role for intrinsic factors such as ovum follistatin and cumulus cell cathepsins in control of ovum quality has led to ongoing research on new technologies to increase yield of transferable embryos.

INTRODUCTION

An elegant review by Keith Betteridge (Betteridge 2003) summarises the history of embryo transfer. The origin of the field of embryo transfer in general dates far back into the 1800's with the pioneering work of Walter Heape who first reported successful embryo transfer in rabbits resulting in live young (Betteridge 2003). Documented insights into the potential application of embryo transfer to animal agriculture were reported 30–40 years later (Betteridge 2003). The first recovery of a bovine embryo (2-cell) was reported in 1931 and the first recorded reports of embryo transfer in farm species (sheep and goats) occurred in

CONFLICTS OF INTEREST

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1932 and 1933 (Betteridge 2003) Important discoveries relevant to superovulation occurred in the 1940s but success in bovine embryo transfer lagged behind (Betteridge 2003). The birth of the first calf resulting from embryo transfer occurred in 1950 at the American Foundation for the Study of Genetics, a private research foundation in Wisconsin, USA founded with business goals focused on commercial application of embryo transfer for propagation of genetics on the female side (Betteridge 2003). These achievement paved the way for future research leading to commercial application of embryo transfer over subsequent decades, including advancements in superovulation, nonsurgical embryo transfer and cryopreservation of embryos, culminating in initiation of commercial bovine embryo transfer in the 1970s (Betteridge 2003).

Bovine embryo transfer as a commercial entity has its origins in North America in the 1970s and was driven by the need for means for multiplication of limited numbers of cows of continental breeds imported into Canada (Mapletoft and Hasler 2005). Statistics compiled by the International Embryo Transfer Society for 2011 revealed that 732,862 transferable embryos were generated *via* superovulation procedures and 572,342 *in vivo* derived embryos were transferred worldwide, with Europe and North America accounting for 19 and 43% of the total embryos transferred (Stroud 2012).

Despite significant interest, progress in production of bovine embryos *in vitro* lagged well behind. *In vitro* fertilisation (IVF) of bovine ova was first reported 36 years ago (Iritani and Niwa 1977), but such procedures required harvest of ova that had reached appropriate stage of maturity *in vivo*. Actually, the birth of the world's first test tube baby in 1978 was likely responsible for rapid acceleration of research into *in vitro* embryo production and development of current technologies for ovum harvest, *in vitro* maturation, fertilisation and embryo culture widely utilised today in both cattle and humans.

The birth of the first calf derived from *in vitro* fertilised embryos (Brackett and others 1982) came three years after the birth of the first test tube baby and spawned significant research since that time. However, application of such technology still lags behind that observed for embryo transfer using *in vivo* derived bovine embryos. Although not widely utilised in North America and Europe, transfer of *in vitro* produced embryos is a growing and viable commercial practice. Statistics indicated that 453,471 transferable *in vitro* produced embryos were generated for commercial application and 343,927 transfers performed in 2011 (Stroud 2012). In contrast to *in vivo* derived embryos, North America and Europe account for only 5.6% and 3.1% of *in vitro* produced embryos transferred, with the vast majority (86.4%) occurring in South America (Stroud 2012). While numbers of *in vivo* produced embryos transferred worldwide have not changed dramatically, transfer of *in vitro* derived bovine embryos has increased by >7 fold since 2000, supporting rapid growth in application of this technology (Stroud 2012).

This review will highlight applications and limitations of embryo transfer using *in vitro* produced embryos in cattle, and recent insight into intrinsic factors that impede efficiency of *in vitro* embryo production. Potential application of such discoveries toward increased efficiency of *in vitro* embryo production will also be discussed.

APPLICATIONS OF EMBRYO TRANSFER USING *IN VITRO* PRODUCED EMBRYOS

A bovine heifer calf is generally born with 10,000 to 350,000 healthy follicles and ova in their ovaries (Erickson 1966), yet a typical beef cow will have 10 or less offspring per lifetime and lifetime offspring numbers are far less in the case of dairy cows. This indicates the total genetic capacity of bovine females in terms of ovum numbers and potential offspring is vastly untapped in a normal production setting. The advent of superovulation procedures provided opportunities for ovulation and fertilisation of multiple ova and harvest of multiple embryos from females beyond the single ovum normally released every 21-day estrous cycle in cattle. Superovulation can clearly increase the numbers of ova ovulated that can potentially be fertilised in a cow's lifetime, but donor cows are typically superovulated only every 65–70d, with the interval shortened to every 40d using prostaglandin and progesterone implant-based protocols to reduce interval between superovulation sessions (Hasler and others 2003). Such protocols can increase total embryos and mean number of embryos per superovulation (Hasler and others 2003). Harvest of ova from cattle *in vivo via* follicle aspiration using ultrasound guided aspiration or ovum pick up procedures (Pieterse and others 1991, Kruip and others 1994, Li and others 2007, Pontes and others 2011, Galli and others 2014), followed by *in vitro* maturation, fertilisation and culture allows for increased frequency of ova collection relative to superovulation as ova can be aspirated twice weekly. Although donor cows produced an average of 4.7 viable embryos per collection using the *in vitro* fertilisation technique and 4.3 viable embryos per collection using the conventional approach, a larger number of embryos per unit time can be generated and transferred using ultrasound guided aspiration and *in vitro* embryo production relative to results using conventional superovulation procedures; (Bousquet and others 1999). Ultrasound guided aspiration and *in vitro* embryo production technologies are particularly widespread in South America where numbers of antral follicles are much higher in native breeds of cattle and yields of embryos obtained higher than obtained using superovulation based procedures (Pontes and others 2009). This likely accounts for the fact that the vast majority of *in vitro* derived embryos transferred occurs in this region of the world.

In vitro embryo production using ova harvested from ovaries collected at a slaughterhouse is common in a research setting for studies focused on improvement of *in vitro* embryo production technologies and elucidating basic mechanisms controlling early embryogenesis. This procedure can yield very large numbers of embryos due to numbers of ovaries available and economy of scale and also holds potential application in a production setting. Typically, the reproductive potential of valuable cows is lost when animals are culled due to disease or other reasons common in a production setting. Hence, *in vitro* embryo production using ova harvested after slaughter in such instances can be used to further propagate the genetic potential of such females (Mapletoft and others 2005).

Use of *in vitro* produced embryos in a commercial dairy industry setting is gaining increased attention and now commercially available. Typically, conception rates in lactating dairy cows are low (<35%) in an intensive production setting which is typical of the United States (Lucy 2001). Such low conception rates can hinder availability of replacement females

from top producing cows. The increase in availability of sex-sorted semen has increased opportunities for dairy producers to better control availability of replacement females. However, conception rates using sex sorted semen are even lower in lactating dairy cows than artificial insemination (42.2 vs 55.6% respectively) with unsorted semen (Schenk and others 2009). This is due most prominently to the reduced numbers of sex-sorted sperm cells packaged per straw, which makes utilisation with conventional artificial insemination less than cost effective. *In vitro* embryo production using a single straw of sex-sorted semen can yield much higher numbers of transferable embryos relative to traditional superovulation coupled with embryo transfer and can also be used to fertilise ova from multiple cows (donors). Thus, *in vitro* embryo production using sex-sorted semen, coupled with embryo transfer, is now being offered by commercial companies for use in lactating dairy cows as a tool for reproductive management to increase numbers of replacement females available.

Other important considerations in potential use of *in vitro* produced embryos derived from sex-sorted or unsorted semen, coupled with embryo transfer, are the potential differences in pregnancy rates for *in vitro* produced embryos relative to pregnancy rates obtained following artificial insemination, and the tolerance of *in vitro* produced embryos to freezing procedures and their reduced utility for use in embryo transfer at a later date. A recent study (Rasmussen and others 2013) revealed lower pregnancy rates in lactating dairy cows following embryo transfer using *in vitro* produced embryos relative to those obtained following timed artificial insemination (28 vs 39.3% respectively). However, no difference in pregnancy rates for *in vitro* produced embryos generated using sex-sorted versus unsorted semen (27.3 vs 30.2% respectively) was observed and expected marked increase in percentage of female calves born from embryos generated using sex-sorted semen occurred (Rasmussen and others 2013). The slightly reduced pregnancy rates observed for embryos generated using sex-sorted semen mirrors differences in pregnancy rates for timed artificial insemination using sex-sorted versus conventional semen (Norman and others 2010). Several previously field studies also reported slightly lower pregnancy rates following embryo transfer for *in vitro* produced embryos versus *in vivo* derived embryos or those obtained following artificial insemination. Day 60 pregnancy rate was 53.4% vs. 59.5% for *in vitro* vs *in vivo* produced embryos respectively (Bousquet and others 1999). In another study overall pregnancy rate was 54% vs 76% for grade 1 day 7 *in vitro* fertilised embryos vs *in vivo* produced embryos respectively (Hasler 2000). Unfortunately, *in vitro* produced bovine embryos are more susceptible to damage during embryo freezing procedures (Lonergan and others 2006), making transfer of fresh rather than frozen embryos the highly preferred approach. Clearly, more research is needed to determine reasons for and solutions to reduced cryotolerance of *in vitro* produced embryos, potential differences in pregnancy rates between *in vitro* and *in vivo* produced embryos following embryo transfer and the mechanisms responsible, and to determine potential economic advantages of increased numbers of replacement females obtained when using embryo transfer with *in vitro* produced embryos generated with sex-sorted semen.

FACTORS THAT LIMIT EFFICIENCY OF *IN VITRO* EMBRYO PRODUCTION

Many variables can influence pregnancy success after transfer of *in vitro* produced bovine embryos including embryo culture system, embryo quality, number of embryos per

recipient, synchrony of embryo development with recipient day of estrous cycle, type of embryo (fresh vs. frozen) and environmental factors such as heat stress (Bousquet and others 1999, Hasler 2000, Farin and others 2001). Many such factors, with the exception of the first listed (embryo culture system), also limit efficiency of embryo transfer using *in vivo* produced embryos and are not the focus of this review. Great strides have been made in increasing efficiency of *in vitro* embryo production relative to when procedures were first developed. However, 65–70% of *in vitro* produced embryos typically do not reach a stage of development or quality where transfer to recipient females is likely to be successful (Lonergan and Fair 2008). The following section will summarise available information on intrinsic factors that influence efficiency of *in vitro* embryo production in cattle as defined by yield of embryos that reach the blastocyst (transferable) stage.

Ovum quality or developmental competence is commonly referred to as the capacity of the ovum to mature, cleave after fertilisation, help promote embryonic development and pregnancy establishment, and bring a pregnancy to term in good health (Krisher 2004, Sirard and others 2006). Elegant experiments designed to compare maturation and fertilisation of ova *in vitro* versus *in vivo* and culture *in vitro* versus *in vivo*, demonstrated that ovum origin or quality is the main factor affecting blastocyst yield for *in vitro* produced embryos. Use of *in vivo* matured ova yielded higher cleavage rates and blastocyst yields following *in vitro* fertilisation than *in vitro* matured ova (Rizos and others 2002). Criteria for prediction of ovum quality however are very limited. Typically, subjective morphological criteria such as ovum shape, homogeneity of cytoplasm and compactness of cells surrounding the ovum (known as cumulus cells) are used to assess ovum quality, and in the final analysis select for and generate embryos of increased developmental potential (Lonergan and others 2003, Coticchio 2004, Krisher 2004) relative to unselected ova. However, amongst the best quality ova selected by morphological criteria, there is considerable variation in capacity to promote embryonic development to a transferable stage after fertilisation and hence a full term pregnancy (Lonergan and others 2003, Coticchio 2004, Krisher 2004). Hence the identification of objective molecular markers of ovum quality is of significant practical application.

Ovum competence is acquired during folliculogenesis as the ovum grows, and during the period of ovum maturation prior to ovulation (Krisher 2004). During early folliculogenesis, ova store specific gene products that will determine their ability to promote follicular growth and the initial cell divisions after fertilisation until control is transferred to products of the embryonic (maternal and paternal) genomes (Bettegowda and others 2008a). Follicle and ovum size seem to be critical factors impacting ovum quality. In general, ova derived from larger sized follicles are more likely to acquire developmental competence and progress to the blastocyst stage following *in vitro* fertilisation than those derived from smaller sized follicles (Blondin and Sirard 1995, Iwata and others 2004, Feng and others 2007). Previous studies demonstrated that ova originating from follicles smaller than 2mm in diameter are more likely to arrest in development following *in vitro* fertilisation, whereas ova obtained from follicles with diameters measuring 2–4mm and >4mm possessed comparable developmental competence (Pavlok and others 1992). Studies demonstrated that the blastocyst yield following *in vitro* fertilisation from ova originating from >6mm follicles was higher than that of ova harvested from 2–6mm follicles (Lonergan and others

1994). The stage of the estrous cycle when collected as well as functional status of the growing follicles also influences the developmental potential of ova following fertilisation (Machatkova and others 1996). In cattle, follicular development occurs in waves (Ireland and others 2000) and the growth phase of the wave prior to dominant follicle selection is more effective for ovum collection as the number of medium sized follicles and the developmental competence or quality of ova from small follicles are decreased during non-growth phase when a dominant follicle is present (Machatkova and others 2004). Furthermore, evidence supports a relationship between ovum size and developmental competence. Ova less than 110μm are believed to still be in the growing phase of development and to have not fully achieved developmental competence. It is right after this time point when acquisition of mRNAs encoding for gene products critical to developmental competence following fertilisation is believed to be complete (Fair and others 1995). This developmental time point is also the basis of a diagnostic test for ovum selection, based on staining with a dye (brilliant cresyl blue) that detects activity of the glucose-6-phosphate dehydrogenase enzyme that decreases as ova reach maximal size (Mangia and Epstein 1975). Use of this test as a basis for ovum selection can increase rates of blastocyst development for *in vitro* produced bovine embryos (Pujol and others 2004, Alm and others 2005), but a large proportion of ova selected using this criteria still fail in development at subsequent stages following *in vitro* fertilisation.

It has been proposed that perturbations in accumulation of specific factors critical to developmental checkpoints in the life of an ovum could be functionally associated with poor ovum quality (Lee and others 2009). Thus, investigation of the molecular characteristics of ova of poor developmental competence is critical to form a foundation for the development of future classification criteria for the selection of ova with superior developmental capacity. A well-defined bovine model for investigation of ovum quality is the prepubertal calf model (Khatir and others 1996, Armstrong 2001). Follicles with fully grown ova are present in calves at or prior to birth, but embryonic development *in vitro* to the blastocyst stage and overall success of pregnancies from embryos derived from ova of prepubertal animals is low (Revel and others 1995). Previous studies from our laboratory (Patel and others 2007, Bettegowda and others 2008b) utilised this model and genomics approaches to identify novel factors in the ovum associated with ovum quality. Results showed lower abundance of mRNA for a protein known as follistatin in poor quality ova harvested from prepubertal calves versus good quality ova harvested from adult cattle (Patel and others 2007). We also found reduced amounts of mRNA and protein for follistatin in 2-cell embryos that cleave late versus embryos that cleave early (Patel and others 2007, Lee and others 2009). Time to first cleavage is a well-established model relevant to ovum quality (Lonergan and others 1999). Bovine embryos that cleave early reach the blastocyst stage at much higher rates than late cleaving embryos (Patel and others 2007, Lechniak and others 2008, Lee and others 2009) and regulation of time to first cleavage is controlled by products of ovum origin prior to activation of the new (embryonic) genome at a later stage of development (Bettegowda and others 2008a).

Based on these results, a functional role for follistatin in regulation of early embryonic development was proposed and studies conducted to determine the ability of exogenous follistatin protein supplementation to promote development of *in vitro* produced bovine

embryos to the blastocyst stage. Addition of exogenous follistatin to bovine embryo culture media increased proportion of embryos that cleaved early, numbers of embryos that reached the blastocyst (transferable) stage and increased the trophectoderm cells number (index of embryo quality) in resulting blastocysts (Lee and others 2009). Trophectoderm cells are cells that go on to form the placenta (Gardner 1989). Complementary loss of function studies also showed that endogenous follistatin is required for embryonic development to the blastocyst stage and a normal complement of trophectoderm cells (Lee and others 2009). Collectively, results suggest that follistatin treatment during *in vitro* embryo culture can be utilised to increase yields of *in vitro* produced embryos reaching the blastocyst stage and quality of such embryos. Furthermore, results of our recent studies (unpublished data) showed that follistatin treatment can enhance the development of cloned (nuclear transfer) bovine embryos to the blastocyst stage and restore a normal complement of trophectoderm cells in the blastocysts. Given cattle pregnancies derived from cloned embryos usually fail and placental development is abnormal in such animals (Arnold and others 2008), it is anticipated that follistatin treatment of cloned embryos during embryo culture may have beneficial effects on placental development and pregnancy success after embryo transfer. The effects of follistatin treatment of cloned and *in vitro* fertilised bovine embryos on pregnancy success following embryo transfer are currently being investigated.

Using the prepubertal ovum model described above, our laboratory also identified molecular markers in the cumulus cells surrounding the ovum that are predictive of bovine ovum competence and elucidated their functional significance (Bettegowda and others 2008b). The mRNAs encoding for a family of proteins (enzymes) known as cathepsins (cathepsins B, S and Z) were elevated in the cumulus cells surrounding poor quality ova harvested from prepubertal calves relative to cumulus cells surrounding good quality ova harvested from adult cattle (Bettegowda and others 2008b). Cumulus cells cathepsin B, S and Z expression was shown retrospectively to be negatively associated with embryonic development to the blastocyst stage (Bettegowda and others 2008b, Warzych and others 2012). Furthermore, inhibition of activity of these enzymes during *in vitro* ovum maturation enhanced rates of development of bovine embryos to the blastocyst stage following *in vitro* fertilisation (Bettegowda and others 2008b, Balboula and others 2010, Min and others 2014). Activity of these proteins (cathepsins) was also negatively associated with health status of the cumulus cells that surround the ovum (Bettegowda and others 2008b). Collectively, results support a negative association and inhibitory effect of cumulus cell cathepsin B, S and Z expression on ovum competence and demonstrate that inhibition of cathepsin activity during ovum maturation can be utilised as a tool to enhance blastocyst rates following *in vitro* fertilisation. These results also indicate that cathepsins B, S and Z expression in the cumulus cells may hold promise as an indicator or novel diagnostic tool to predict the capacity of bovine ova to support development to the blastocyst stage following fertilisation, an early indicator of pregnancy potential.

In vivo ova and preimplantation embryos are exposed to a mixture of growth factors, secreted by follicles and or the oviducts and endometrium (Richter 2008). Numerous *in vitro* studies have been previously conducted to elucidate the effects of other potential trophic factors, including Insulin like growth factor-1 (IGF-1), colony stimulating factor 2 (CSF-2), and fibroblast growth factor 2 (FGF-2) on ovum and or embryo developmental competence

and post transfer survival of resultant embryos. *In vitro* bovine ovum maturation (Herrler and others 1992, Moreira and others 2002) or embryo culture (Sirisathien and others 2003) in the presence of IGF-1 increases the proportion of embryos developing to the blastocyst stage. IGF-1 also enhances embryo competence for post-transfer survival when heat stressed lactating dairy cows are used as recipients. (Block and others 2003, Block and Hansen 2007, Hansen and others 2010). Transfer of embryos cultured in the presence of IGF-1 results in higher pregnancy rates at day 21 and less pregnancy loss between day 21 and 30 than observed for untreated control embryos (Block and Hansen 2007). Similarly, heat stressed recipients that received embryos cultured with IGF-1 have greater pregnancy rates at Days 30–35 and reduced pregnancy loss between Days 30–35 and term than recipients that received untreated control embryos (Loureiro and others 2009). Results support positive effects of exogenous IGF-1 on oocyte/embryo competence in cattle.

Beneficial effects of FGF-2 and CSF-2 supplementation during *in vitro* embryo culture have also been reported. FGF-2 supplementation to culture media increases bovine blastocyst formation rates *in vitro* (Lee and Fukui 1995, Fields and others 2011), with increased inner cell mass cell number and a higher ratio of ICM to trophectoderm cells (de Moraes and Hansen 1997, Loureiro and others 2009). Loureiro and others 2009 conducted two experiments to determine the effect of CSF-2 supplementation during *in vitro* embryo culture on post-transfer survival of bovine embryos. Addition of CSF-2 between day 1–7 had no effect on pregnancy rate whereas CSF-2 supplementation between day 5–7 decreases pregnancy loss between day 30–35 of gestation and term and increases calving rates (Loureiro and others 2009). Results support trophic actions of CSF-2 and FGF-2 on *in vitro* bovine early embryonic development and of CSF-2 on post transfer development of bovine embryos.

CONCLUSIONS

Although over 60 years have passed since the birth of the first calf resulting from embryo transfer, widespread use of embryo transfer technology beyond the seed stock industries remains limited. The use of *in vitro*-produced embryos for embryo transfer, while much more common in South America, is even less prominent than traditional embryo transfer in Europe and North America. New applications for such technologies are emerging, such a coupling use of sex selected semen with *in vitro* embryo production and embryo transfer in lactating dairy cows to increase numbers of females born. Strategies incorporating *in vitro*-produced embryos can more efficiently utilise sex-sorted semen than is possible using artificial insemination. Efficiency of *in vitro* embryo production and reduced cryotolerance limits wider application of embryo transfer using *in vitro* derived embryos and increases resulting costs. Recent research into intrinsic factors influencing bovine ovum quality holds application potential to increase efficiency of *in vitro* embryo production, but more research is needed to determine effects of treatments developed on pregnancy success.

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