Using basic approaches to address applied problems in dairy reproduction

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Poor reproductive efficiency is a worldwide problem affecting the dairy industry. There is substantial evidence for an association between high milk production and lower conception rates observed in cows compared to heifers. However, whether the decline in fertility is due directly to the level of milk production or other factors associated with lactation is unclear. There are various checkpoints along the developmental axis which could, in part, contribute to reduced fertility including suboptimal follicle development associated with poor oestrus exhibition, suboptimal oocyte quality, altered sperm transport and fertilization and/or a suboptimal reproductive tract environment incapable of supporting normal embryo development. The challenge is deciphering where the major problems lie. Evidence for the relative contributions of oocyte quality, embryo quality and the reproductive tract environment is discussed in this paper.

Introduction and context

Poor reproductive efficiency is a worldwide problem affecting the dairy industry and has been the subject of numerous excellent reviews (e.g., Lucy 2001, Inskeep & Dailey 2005, Leroy *et al.* 2008a,b, Inskeep & Dailey 2010). While in some locations this situation is exacerbated by problems of heat stress during summer (Hansen 2007), even in more moderate climates a steady decline in fertility has been noted. There is substantial evidence for an association between high milk production and the lower conception rate observed in cows (25-40%) compared to heifers (60-75%) (Sreenan & Diskin 1986, Pursley *et al.* 1997, Lucy 2001, Diskin & Morris 2008). However, whether the decline in fertility is due directly to the level of milk production or other factors associated with lactation is unclear (Chebel *et al.* 2008).

Infertility in dairy cattle is a multifactorial problem which may be linked to various checkpoints along the developmental axis including suboptimal follicle development associated with poor oestrus exhibition, suboptimal oocyte quality, altered sperm transport and fertilization and/or a suboptimal reproductive tract environment incapable of supporting normal embryo development. One of the obstacles to achieving a better understanding of the causes of poor fertility is the difficulty in separating these various issues from each other. For example, while the proportion of pregnancies after AI declined in past 50 years, there has been little change in the average number of oocytes/embryos and transferable embryos produced in embryo transfer (ET) programmes in USA/Canada; furthermore, there has been little change in the proportion of animals that become pregnant following ET over a period of approximately 30 years (Hasler 2006).

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Approaches to understanding embryo mortality – when does the problem occur?

One approach to identifying at which stage along the developmental axis problems arise has been to inseminate animals and either recover embryos at given stages after insemination to determine fertilization failure and timing of embryonic mortality, or to continually monitor pregnancy to pinpoint the period of embryonic loss. Published data indicate fertilisation rates of 90% and average calving rates of about 55% are normal for heifers and moderate yielding dairy cows, indicating an embryonic and foetal mortality rate of about 35% (Sreenan & Diskin 1986, Diskin *et al.* 2006, Diskin & Morris 2008). Relatively few embryos are thought to be lost between fertilization and Day 8 of gestation (corresponding to the blastocyst stage); 70-80% of the total embryonic loss is estimated to occur between Days 8 and 16 after insemination (corresponding to the day of maternal recognition of pregnancy in cattle); a further 10% between days 16 and 42, by which time implantation is complete, and further 5-8% between day 42 and term.

Dunne *et al.* (2000) reported that embryo survival rates in beef heifers on Days 14, 30 and at full-term were similar (68%, 76%, 72%, respectively), indicating that most embryo loss, at least in beef heifers, occurs before Day 14. Silke *et al.* (2002) reported embryonic loss of 6-7% between Day 28 and 84 of gestation in dairy cows and heifers. Starbuck *et al.* (2004) reported embryonic loss of about 11% between Day 30-60 and related this loss to concentration of progesterone at week 5 of gestation, twin ovulation, body condition, age and sire. Both of these studies provide evidence for a relatively low incidence of late embryonic loss.

The sparse data from flushed early embryos from normally ovulating (i.e., non-superovulated), high-yielding lactating dairy cows indicate that fertilization rate is also high (83%, 610/732 in review by Sartori *et al.* 2010), but few studies have directly compared lactating and nonlactating dairy cows. In the study of Sartori *et al.* (2002), comparing lactating and nonlactating (either nulliparous heifers or dry cows) Holstein cattle, fertilization was only reduced during summer in lactating dairy cows; however, lactating dairy cows had poorer embryo development than nonlactating females, irrespective of season. This last observation is interesting as it suggests that the ability of the reproductive tract to support normal embryo development may be impaired in lactating cows. However, oocyte quality cannot be ruled out as a contributing factor, as it is clear from IVF studies, where typically 80% of inseminated oocytes cleave and 30-40% develop to blastocysts, that fertilization success is no guarantee of future development (Lonergan 2007).

Sartori *et al.* (2010) reviewed data on fertilisation and embryo quality up to Day 7 post AI in single-ovulating and superovulated dairy cows. The authors concluded that fertilisation was not the primary factor reducing viable embryo yield in embryos recovered from non-heat stressed single-ovulating, lactating dairy cows. In contrast, fertilisation failure appeared to be a major cause of reduced embryo yield in superovulated lactating (57%, n=8938) and nonlactating (50%, n=16039) dairy cows (Sartori *et al.* 2010). Summarising recent data (Wiebold 1988, Ryan *et al.* 1993, Sartori *et al.* 2002, Cerri *et al.* 2009a,b,c) the authors concluded that $<50\%$ of ova and/or embryos recovered from high-yielding dairy cows are viable 7 days after AI (Sartori *et al.* 2010).

Chebel *et al.* (2008) evaluated factors affecting success of on-farm ET programmes in large dairy herds. Non-lactating dairy cows had a greater response to superovulation, yielded a greater proportion of ova/embryos, an increased number of fertilized oocytes and an increased number of viable embryos compared to lactating dairy cows.

Isolating follicle/oocyte issues – the argument for a guilty oocyte

Following parturition, in association with peak milk yield, high yielding dairy cows enter a variable period of negative energy balance where the energy expenditure for peak milk production is not matched by the energy derived from dry matter intake. During this period of energy deficit, fats are mobilized from endogenous stores resulting in, among other things, increased non esterified fatty acids in circulation and in follicular fluid which has been associated with poor oocyte quality (Leroy *et al.* 2005).

One way of experimentally separating potential issues surrounding the follicle and/or oocyte from issues relating to the reproductive tract environment is to use transvaginal ovum-pick up coupled with IVF. Differences in development seen in this scenario would by definition not be related to post-ovulation issues, i.e., the reproductive tract, but rather reflect the intrinsic quality of the oocyte. While several authors have reported development of ovum-pick up/IVF embryos in dairy cows, e.g., Fouladi-Nashta *et al.* (2007), few have compared development from lactating dairy cows and non-lactating cows or heifers. In one study from our group (Rizos *et al.* 2005) there was no difference in the proportion of good quality oocytes undergoing fertilization and development to the blastocyst stage between lactating cows and heifers. Snjiders *et al.* (2000) found that a lower proportion of oocytes recovered from dairy cows with a higher genetic merit for milk production underwent cleavage or developed to the blastocyst stage in vitro than those from cows of average genetic merit.

Using ovum-pick up and assessment of oocyte morphology, several studies from Virginia (Kendrick *et al.* 1999, Gwazdauskas *et al.* 2000, Walters *et al.* 2002) have demonstrated that conditions related to early lactation have a negative effect on oocyte quality and endocrine measures in dairy cattle. For example, Kenderick *et al* (1999) conducted OPU twice weekly between Day 30 and 100 of lactation and reported that a low energy diet reduced milk yield, BCS and serum P4 and had a negative impact on oocyte quality.

Isolating embryo/reproductive tract issues – the argument for a guilty embryo/reproductive tract

A series of studies from our laboratory and others involving culture of zygotes in vitro or in vivo from the zygote to blastocyst stage have shown that postfertilization culture conditions do not significantly alter the proportion of in vitro derived bovine zygotes developing to the blastocyst stage but can significantly affect the quality of these embryos (Enright *et al.* 2000, Rizos *et al.* 2002, Lonergan *et al.* 2003). Thus, to a large extent, the quality of the oocyte (which will of course itself be affected by the follicular environment in which it develops) dictates its own developmental fate. This is not to say that an excellent quality oocyte can overcome a deleterious uterine environment.

The use of ET technology allows the endogenous oocyte of the cow to be removed as a confounding factor in understanding the cause of infertility and thus bypasses the events of follicle development and oocyte quality, as well as the potential negative effects of lactation associated metabolic stress on these processes. Several studies from Florida have compared ET with AI in order to overcome poor conception rate of lactating dairy cows due to heat stress (Putney *et al.* 1989, Ambrose *et al.* 1999, Drost *et al.* 1999, Al-Katanani *et al.* 2002; reviewed by Rutledge 2001), the notion being that transferring embryos to recipients at a stage when they are less susceptible to heat stress (i.e., on Day 7) may enhance pregnancy rates during periods of heat stress when the cow's endogenous oocyte would be susceptible. In all of these studies, conception rate was higher for ET than AI when fresh or frozen in vivo produced embryos were used; transfer of frozen (Ambrose *et al.* 1999, Drost *et al.* 1999) or vitrified (Al-Katanani

et al. 2002) IVF embryos had no advantage over AI. Consistent with this, Vasconcelos *et al.* (2006) examined the factors affecting pregnancy rate after ET of in vivo derived fresh embryos to lactating dairy cow recipients. Pregnancy rate, corrected for cows with a CL, were 36.5% (84/230) vs 58.7% (91/155) on Day 25 and 33% (76/230) vs 45.8% (71/155) on Day 46 for AI and ET, respectively. Furthermore, Demetrio *et al.* (2007) reported higher conception rates in lactating Brazilian dairy cows following the transfer of a fresh embryo derived from nonlactating cows compared to after AI. In contrast, Sartori *et al.* (2006) compared ET with AI in dairy cows in Wisconsin at cooler times of the year and found no difference in conception rate. Taken together, these data would suggest that the oocyte is more susceptible to adverse conditions imposed by heat stress and perhaps negative energy balance than the embryo.

Routine ET, such as described above, involves the placement of an embryo, typically on Day 7, into the uterine horn, thus bypassing the oviduct. Up until relatively recently, the oviduct was inaccessible without resorting to major surgery. The recent refinement of endoscopy techniques to allow access to the oviducts of cattle have greatly facilitated the study of early embryo development (Havlicek *et al.* 2005, Wetscher *et al.* 2005, Besenfelder *et al.* 2008). Now it is possible to transfer large numbers of in vitro produced embryos to the oviducts to study development in vivo as well as recovering embryos at specific early stages of development. In studies on beef heifers where 50 – 100 embryos have been transferred to the oviduct ipsilateral to the CL (Tesfaye *et al.* 2007), recovery rates have been in the order of 80%; blastocyst yields have been in the order of 40-50%, in line with what is expected from in vitro derived zygotes.

Until recently, this model of multiple ET had not been attempted in the postpartum dairy cow. We hypothesized that part of the difference in fertility between heifers and post partum lactating dairy cows could be explained by differences in the ability of the reproductive tract (oviduct and uterus) to support early embryo development and that this would be related to circulating progesterone concentration. Testing this hypothesis in single-ovulating animals would be extremely challenging due to the numbers of animals required. Therefore, using endoscopy, we transferred 1800 in vitro produced embryos to the oviducts of nulliparous Holstein-Friesian heifers and post partum lactating Holstein-Friesian cows and assessed their development to the blastocyst stage following recovery on Day 7 (Rizos *et al.* 2010). Recovery rate was lower from cows (57%) compared to heifers (79%) and of the structures recovered only 18% had developed to the blastocyst stage in cows compared to 34% in heifers, providing evidence for an impairment in the ability of the reproductive tract of the post partum cow to support embryo development.

Hasler (2006) reviewed data on embryo production following superovulation in Holstein cattle and subsequent pregnancy rates following fresh ET into Holstein cows and heifers over a 20-25 year period to determine whether the documented decline in fertility of dairy cows has been accompanied by a corresponding decline in the efficacy of superovulation and ET. Little change was reported in either the mean number of transferable embryos resulting from superovulation (which could be interpreted as a proxy for oocyte quality) or in the proportion of recipient animals that became pregnant following ET, although heifers had a higher pregnancy rate than lactating cows. This would suggest that as milk yield has increased over the past several decades, the uterine capacity to support a pregnancy has not changed dramatically and would point to oocyte quality as a significant factor in the decline in dairy cow fertility. Consistent with this, Sartori *et al.* (2002) compared embryo quality on Day 5 from normally ovulating lactating vs nonlactating dairy cows; although fertilization rate was similar (88-90%) the proportion of viable embryos was much lower in lactating cows (53%) than in non-lactating cows (82%).

The ability to separate the contribution of the embryo and the recipient to embryonic survival up to Day 60 of pregnancy has been developed in a model by McMillan (1998) which suggests that variation in recipient quality rather than embryo quality is a major source of variation in pregnancy rates after ET. Based on pregnancy rates after repeated ET, two herds were established ('high' and 'low' pregnancy); significantly more embryos were elongated at Day 14 in the high herd (67%) than the low herd (14%) and this delay was still apparent by Day 17, encompassing the critical window of maternal recognition of pregnancy. Based on subsequent experiments they concluded that the difference in fertility was due to significantly more interferon-tau being secreted by the conceptus acting on a uterus with a reduced capacity to secret prostaglandin, perhaps enabling a wider window for maternal pregnancy recognition.

Isolating embryo/reproductive tract issues – the argument for a guilty reproductive tract and the role of progesterone

The steroid hormone progesterone plays a key role in the reproductive events associated with pregnancy establishment and maintenance. High concentrations of circulating progesterone in the immediate post-conception period have been associated with an advancement of conceptus elongation, an associated increase in interferon-tau production and higher pregnancy rates in cattle (Mann & Lamming 1999, Mann & Lamming 2001, Stronge *et al.* 2005, McNeill *et al.* 2006, Carter *et al.* 2008, Forde *et al.* 2009) and sheep (Ashworth *et al.* 1989, Satterfield *et al.* 2006).

The effects of elevated progesterone shortly after conception on the advancement of conceptus elongation have been convincingly demonstrated in cattle and sheep. Garrett *et al.* (1988) administered 100 mg progesterone on Days 1, 2, 3 and 4 of pregnancy which resulted in an increased peripheral plasma progesterone concentration on Days 2 to 5 and significantly larger conceptuses on Day 14. Using a progesterone implant on Day 3 of pregnancy, Carter *et al.* (2008) significantly elevated progesterone concentrations until Day 8 and this was associated with a larger conceptus recovered at slaughter on Day 16. Similarly, when ewes received daily injections of 25 mg progesterone from 36 h postmating, blastocyst diameter increased by 220% on Day 9 and the time of elongation of blastocysts to a filamentous conceptus on Day 12 was advanced (Satterfield *et al.* 2006); these effects of progesterone treatment on blastocyst development were blocked by administration of RU486, a progesterone receptor antagonist.

From the above, it is clear that the concentration of circulating progesterone has an effect on the developing embryo. This effect is likely as a result of downstream effects of progesterone-induced changes in gene expression in the tissues of the uterus (Bauersachs *et al.* 2006, Satterfield *et al.* 2006, Forde *et al.* 2009) resulting in changes in the composition of histroph to which the developing embryo is exposed. The importance of histotroph for conceptus development was demonstrated in the uterine gland knockout (UGKO) model in sheep in which embryos fail to develop beyond the blastocyst stage in adult UGKO ewes (Spencer *et al.* 2007).

In a series of recent experiments using in vitro and in vivo models we addressed the issue of whether the effects of progesterone on conceptus elongation could be due, at least in part, to a direct effect of progesterone on the embryo (Clemente *et al.* 2009). Progesterone receptor mRNA was present at all stages of embryo development raising the possibility of a direct effect of progesterone on the embryo. Exposure to progesterone in vitro in the absence or presence of oviduct epithelial cells did not affect the proportion of embryos developing to the blastocyst stage, blastocyst cell number or the relative abundance of selected transcripts in the blastocyst. Furthermore, exposure to progesterone in vitro did not affect post-hatching elongation of the

embryo following transfer to synchronised recipients and recovery on Day 14. In contrast, transfer of in vitro derived blastocysts to a uterine environment previously primed by elevated progesterone resulted in a 4-fold increase in conceptus length on Day 14. These data provide clear evidence to support the hypothesis that progesterone-induced changes in the uterine environment are responsible for the advancement in conceptus elongation reported previously in cattle and that, interestingly, the embryo does not need to be present during the period of high progesterone in order to exhibit advanced elongation. This is consistent with the fact that administration of progesterone early in the oestrous cycle can advance uterine receptivity for the transfer of older asynchronous embryos (Geisert *et al.* 1991).

Most of these bovine data on the role of progesterone have been generated in nonlactating beef heifers; for example, there are few if any published reports of endometrial gene expression in the lactating dairy cows despite evidence for suboptimal progesterone concentrations in such animals and evidence for increased progesterone metabolism in the liver (Sangsritavong *et al.* 2002). The critical involvement of oestradiol and progesterone in almost every aspect of reproductive physiology makes changes in steroid metabolism an attractive explanation for the numerous changes in reproduction in lactating dairy cows. Similar data on endometrial function in the dairy cow would aid our understanding of factors associated with embryonic mortality.

A number of treatments can be used to increase peripheral concentrations of progesterone after AI including those that increase endogenous function of the existing CL, induce accessory CL, or supplement progesterone directly (see reviews by Lamb *et al.* 2010, Binelli *et al.* 2001, Inskeep 2004). However, data on outcome in terms of pregnancy rate are often conflicting and may reflect timing of treatment as well as the fact that only a proportion of animals with inherently low progesterone may benefit from such treatment. Stevenson *et al.* (2007) assessed effects of a variety of interventions after AI on fertility including administration of GnRH, hCG or an intravaginal progesterone-releasing device (CIDR). GnRH and hCG effectively induced ovulation and increased CL number but only increased circulating progesterone concentrations in hCG-treated cows. Treatment with a CIDR or hCG increased conception rate but only in some herds.

So, is it all down to the endometrium?: maternal-embryonic cross-talk

The ability to transfer an in vitro derived embryo (i.e., one developed in the absence of any contact with the female reproductive tract) to a synchronized recipient and obtain acceptable pregnancy rates would suggest that the embryo is somewhat autonomous for at least the first week of life and that direct contact with the maternal reproductive tract is, to a certain extent, unnecessary.

In support of this, when we compared the transcriptome of the endometrium in pregnant and cyclic heifers on various days from oestrus (Day 5, 7, 13 and 16) we could only detect differentially expressed genes in the endometrium on Day 16, coincident with a filamentous embryo secreting large amount of interferon-tau (Forde *et al.* 2010). This would suggest that the cow, or more specifically her uterus, is always an optimist regarding likelihood of pregnancy i.e., that the temporal changes occurring in the endometrium are similar in pregnant and cyclic cows up the point when luteolysis normally occurs.

The origin of embryo (e.g., in vivo derived following superovulation *vs* in vitro produced following IVF *vs* nuclear transfer) can have a significant impact on the dynamics of embryo mortality. Heyman *et al.* (2002) monitored the evolution of pregnancy following the transfer of embryos derived from somatic cell cloning, embryonic cloning and IVF in order to detect

the occurrence of late gestation losses and their frequency. On the basis of progesterone concentrations on Day 21, there were no significant differences in the percentages of initiated pregnancies between the groups (55.6-62.7%). Confirmed pregnancy rate by Day 35 using ultrasound scanning was significantly lower in the two somatic cloned groups (27.5-33.8%) compared with the embryonic clones (49.2%) and IVF embryos (52.9%). This pattern was maintained at Days 50, 70 and 90. The incidence of loss between Day 90 of gestation and calving was 43.7% for adult somatic clones and 33.3% for foetal somatic clones compared with 4.3% after embryonic cloning and 0% after IVF.

Two recent key papers provide strong evidence that the endometrium of the cow reacts differently to different embryo types (Bauersachs *et al.* 2009, Mansouri-Attia *et al.* 2009); in other words, embryos of different quality (i.e., with divergent developmental fates) signal differently to the endometrium and in turn elicit a different response in terms of the transcriptome of the endometrium. In this way, the endometrium can be considered as a biological sensor able to fine-tune its physiology in response to the presence of embryos whose development will become altered much later after the implantation process (Mansouri-Attia *et al.* 2009).

Conclusion

Many factors are likely to impact on the success or otherwise of pregnancy. It is clear that maternal factors (e.g., oocyte quality, reproductive tract environment) can have a strong influence on the likelihood of embryo survival. However, the inherent quality of the embryo can also affect the likelihood of it undergoing early embryonic mortality, for example, by failing to elicit the correct response from the endometrium to ensure an optimal environment.

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