THE ESSENCE OF FERTILIZATION: OOCYTE MEETS SPERM

De essentie van de bevruchting: de ontmoeting tussen de eicel en de zaadcel

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ABSTRACT

The problem of reduced fertility in high yielding dairy cattle is a very complicated one, and the relationship between various measures of fertility and level of milk production remains controversial. In this brief review the essence of the problem is considered: what is the oocyte's and the sperm's contribution, and what is the importance of the resulting embryo in the declining fertility of the Holstein Friesian cow?

SAMENVATTING

Het probleem van de verminderde fertiliteit bij hoogproductief melkvee is zeer complex, en het verband tussen verschillende fertiliteitsparameters en de melkproductie blijft in vele gevallen controversieel. In dit korte overzicht wordt getracht de essentie van het probleem te behandelen: welke rol spelen de eicel, de zaadcel en het gevormde embryo in de tanende vruchtbaarheid van de Friese Holsteinkoe?

INTRODUCTION

The earliest known remains of domesticated cattle, dating to 6,500 B.C., were found in Turkey, and findings on other sites in the Near East also approach this age. Early domestic cattle were bred for a triple purpose: for use as draught animals, and for dairy and meat production. Later, in Western Europe, cattle were replaced by horses for draught purposes, and much later by machinery. From the 20th century on, cattle breeds have mainly been selected for a dual purpose: milk and meat. During the last fifty years, however, selection has been more directed towards extremes, leading to specialized breeds such as the Holstein Friesian, which can yield averages of up to 12,000 liters of milk per year, and the Belgian Blue, a hypermuscular breed bred especially for meat production. These are the two predominant cattle breeds in Belgium. The excessive specialization which occurred in Holstein Friesian and Belgian Blue cattle has led to major fertility problems in both breeds, though for different reasons in each. The fertility problems in high yielding dairy cattle are especially a reason for concern, since they may be more difficult to solve than the fertility problems in the Belgian Blue (Leroy and de Kruif, 2006).

How did these fertility problems originate in the first place? Ruminants are under general circumstances quite fertile animals. An extreme example of ruminant fertility is the reindeer, in which 99.5 % of matings lead to viable fetuses and, under ideal conditions, fawn crops may reach 85-95 % (Godkin, 1986). In domesticated dairy cattle, which is mostly bred by artificial insemination, satisfactory pregnancy rates are situated between non-return rates of 65 to 70 %, leading in only 50-55 % of the cases to the birth of live calves. In high yielding dairy cattle, fertility levels have dropped to 40 % (Leroy and de Kruif, 2006). What is the underlying cause of this problem? In fact, fertilization is such a complicated process that there are numerous causes for fertility problems and, if you think of it, it is a miracle that any offspring should be produced in the first place!

In order for a live calf to be born, a number of well-orchestrated events need to take place in timely order, and any error in this sequence of events leads to the prevention of fertilization or to the untimely death of the conceptus. In this series of events I will not consider either the impor-

tance of estrus detection by the farmer or the importance of the timing of insemination, both of which have been covered by other authors (see Roelofs *et al.*, 2006 and Van Eerdenburg, 2006). First of all, in the days before estrus a healthy oocyte has to develop in a dominant follicle, followed by the final events of nuclear and cytoplasmic maturation during estrus, leading to ovulation of this single oocyte on average 12 h after the end of estrus. At the time of ovulation, a limited number of capacitated sperm at the site of fertilization in the Fallopian tube is sufficient to allow for fertilization of the oocyte to occur. The fertilizing sperm has to bind to and subsequently penetrate the zona pellucida, merge with the oolemma and trigger oocyte activation and further embryo development. In cattle, the resulting zygote will then go through a series of cleavage divisions while residing in the oviduct, and after less than one week it will arrive in the uterus. After compaction, which occurs at about day 6 after insemination, bovine embryos can be flushed from the uterus and either be transferred into a recipient cow, frozen or used for research purposes. Under normal conditions, however, the embryo remains in the uterus, goes through the blastocyst stage, and hatches from the zona pellucida. At this stage, the embryo becomes implantation competent, but in cattle, implantation is not completed until 30 days after fertilization (reviewed by Lee and De Mayo, 2004). After implantation, a host of causes, such as chromosomal, placental, or ovarian/uterine abnormalities (see Thatcher *et al.*, 2006), may induce embryonic or fetal mortality, but this aspect will not be discussed here.

It has been stated that the environment experienced by the embryo and fetus *in vitro* and *in utero* not only affects the chances for live offspring, but also produces effects that persist into adulthood and subsequent generations (Betteridge, 2001). This vulnerability of the embryo to its early environment has become obvious to those scientists who have been transferring embryos from *in vitro* fertilization and cloning, and whose efforts have been plagued by early embryonic losses and by the "Large Offspring Syndrome" (Betteridge, 2006). Early embryonic loss is also a problem in lactating dairy cattle (Thatcher *et al.*, 2006), but the occurrence of calves that are larger than normal has not been described in them so far. However, in high yielding dairy cattle the parallel with *in vitro* cultured embryos is valid to that extent that cattle embryos sojourning in the genital tract of a lactating cow (and oocytes and sperm before them) are also exposed to unfavorable conditions. The objectives of the present review are: (1) to summarize data from older and more recent studies in order to gauge the influence on the resulting conceptus of metabolic and nutrient changes experienced by the oocyte; (2) to draw attention to the second (and somewhat neglected) player in fertilization, the spermatozoon, and whether its arrival at the sperm reservoir may be jeopardized; and (3) by making use of data generated by reproductive techniques, both by means of *in vitro* and *in vivo* studies, to consider the importance of changes in the culture medium or in the oviductal/uterine environment for the developing preimplantation embryo.

OOCYTE VULNERABILITY

The oocyte is the rarest and the largest cell in the body of a dairy cow and, although about 200,000 primordial follicles are present in the ovaries of a newborn heifer calf, only about 150 oocytes will have reached the ovulatory stage in a ten year old cow. The oocyte waits for years before beginning to grow, and not until this process is complete can it resume meiosis and undergo fertilization (Gosden *et al.*, 1997). The cumulus oophorus, which is unique to the egg of eutherian mammals (Bedford and Kim, 1993), is surrounding the oocyte in the follicle. Cumulus cells are involved in oocyte growth and maturation and are important for supplying the oocytes with nutrients and connecting them to the external world (Tanghe *et al.*, 2002). The vascular supply of the follicle is situated in the theca interna (Redmer and Reynolds, 1996), which means that in cattle, mature oocytes can be located at a considerable distance from the vascular supply of oxygen, nutrients and signals. This distance is bridged by a gap junction network between the cumulus cells that are making contact with the growing oocyte by means of transzonal cumulus processes, which allow for a quick transfer of small metabolites and regulatory molecules into the oocyte. In fact, this unique feature of cumulus cells, together with their specific metabolizing capacity, is of capital importance in the regulation of oocyte maturation (Tanghe *et al.*, 2002). It is therefore not surprising that changes brought about in the follicular fluid or in the vascular supply surrounding the follicle can have major impacts upon the oocyte. It has been shown in humans, for instance, that cigarette smoking, which has been associated with accelerated follicular depletion and derangement of reproductive functions, may be causing increased oxidative stress in the follicle (Paszkowski *et al.*, 2002). Evidence for this hypothesis is given by the finding that female smokers have increased levels of lipid peroxidation in their follicular fluid (Paszkowski *et al.*, 2002), possess higher numbers of apoptotic cumulus cells (Sinko *et al.*, 2005) and show follicular depletion of the anti-oxidant beta-carotene (Tiboni *et al.*, 2004) compared to non-smokers. Our non-smoking high producing dairy cows are equally subject to environmental, nutritional and metabolic aberrations which may interfere with oocyte quality and maturation. *In vivo* studies, in which a serial follow-up of dairy cattle in a negative energy balance is being performed by means of repeated transvaginal follicle

puncture, yield valuable information on changes in follicular fluid which are a reflection of the metabolic status of these animals (Leroy *et al.*, 2004). Moreover, such data may serve as a basis for an *in vitro* model, in which important substances or metabolites can be added to the *in vitro* maturation medium in order to study their effects on bovine oocyte quality. This approach is a simplification of the situation *in vivo*, in which it may take 180 days for a primordial and 80 days for an antral follicle to reach the preovulatory stage (Campbell *et al.*, 1995). Oocytes which are developing in lactating cattle during a period of negative energy balance may therefore be subjected to unfavorable conditions during this complete time period, whereas *in vitro* matured oocytes are only cultured in the presence of a presumptive noxa. However, since bovine primordial follicle culture is still not optimal, *in vitro* maturation of cattle oocytes is the best available model at present. Using this approach, insulin, IGF-I, growth hormone, leptin, urea, ammonia and non-esterified fatty acids have all been implied as important determinants for oocyte quality in high-producing dairy cattle (Armstrong *et al.*, 2003; Jorritsma *et al.*, 2003) (Table 1).

From these data it appears that some metabolites which are present in the follicular fluid of lactating dairy cattle may have a negative influence both on the oocyte and on its subsequent embryonic developmental capacity. We have come to appreciate the vulnerability of the oocyte to its microenvironment by investigating not only the immediate effect of biological substances on oocyte maturation, but also the effects which are situated at a some what further distance in time. The first effect of a noxious substance which becomes obvious is at the level of nuclear maturation, with a decreased progression of the oocyte from prophase I to the metaphase II stage. For *in vitro* maturation, immature oocytes in the germinal vesicle stage (prophase of meiosis) are aspirated from 3-8 mm antral follicles. Putting these oocytes in the right culture medium induces in 90 % of them the resumption of meiosis (metaphase II stage). The second effect is reduced fertilization and cleavage rates of the resulting embryos. The third effect is the drop in blastocyst rates (Leroy *et al.*, 2005a). These far-reaching consequences are quite logical, given the fact that the oocyte cytoplasm is filled with transcripts and proteins that are necessary for early embryonic development (Schultz, 2002). The first embryonic cleavage divisions can proceed based upon these maternal proteins derived from the oocyte cytoplasm. Changes in oocyte quality may therefore affect both cleavage divisions and blastocyst yield. During follicular growth, immature oocytes transcribe and store important mRNAs and proteins necessary for embryo development. On attainment of the oocyte's full size (which is reached in

Table 1. Studies in which the addition of compounds/metabolites present in higher concentrations during the period of negative energy balance in lactating dairy cattle showed an effect upon oocyte maturation and/or embryo development.

Compound	Effect on oocyte maturation	Effect on embryo developmental capacity	Reference
Leptin	Positive	Positive	Boelhauve *et al.*, 2005
Palmitic acid	Negative	Negative	Leroy *et al.*, 2005a
Stearic acid	Negative	Negative	Leroy et al, 2005a
Oleic acid	No effect	No effect	Leroy *et al.*, 2005a
Urea	Negative	No effect	De Wit *et al.*, 2001
Ammonia	Negative*	NA[£]	Rooke *et al.*, 2004
Growth hormone	Positive	Positive	Izadyar *et al.*, 1996
Insulin	No effect	NA	Matsui *et al.*, 1995
IGF-I	Positive	NA	Lorenzo *et al.*, 1994

*Ovine oocytes
[£]NA: Not analyzed

a 3 mm bovine follicle), transcription ceases and the mRNA that was previously stored must then drive development through oocyte maturation, fertilization and the early cleavage stages up to the activation of the embryonic genome (Fair *et al.*, 2004). These maternal transcripts may also participate in embryonic genome activation, which takes places at the 8 to 16 cell stage in the bovine embryo (Vigneault *et al.*, 2004). It has been shown that optimal maturation conditions, such as are happening during *in vivo* maturation, increase the blastocyst yield significantly, which demonstrates that the intrinsic quality of the oocyte is the main factor determining blastocyst yield (Rizos *et al.*, 2002).

From the above, it is obvious that the oocyte is a very important player in the process of fertilization and embryo development: there are sufficient data to suspect that oocyte quality is indeed impaired in lactating dairy cattle.

SPERM FATE

Most dairy cattle (over 95%) are inseminated with frozen-thawed sperm. Depending on bull fertility, sperm numbers which are being deposited into the uterine body vary between 5 and 15 million (Den Daas *et al.*, 1998). Due to the leukocytic infiltration of the uterine cavity which takes place in response to insemination, the uterus represents a rather hostile environment for spermatozoa and rapid sperm transport through this anatomical part of the female genital tract enhances sperm survival. This sperm transport is mainly effected by myometrial contractions, whereas the swimming speed of the sperm itself is probably of minor importance. The distance between the uterine body and the uterotubal junction may be about 20 cm in adult cows: it should take about half an hour to traverse the uterine cavity for sperm swimming at about 6.5-7 mm/min, which is the average swimming speed of frozen-thawed Holstein spermatozoa as measured *in vitro* by Computer Assisted Sperm Analysis (Hoflack *et al.*, 2006). During the hours before ovulation, motile sperm are directed towards the sperm reservoir which, in cattle, is located at the uterotubal junction. At this specific place, mucosal folds form cul-de-sacs with openings facing backwards towards the uterus (Yaniz *et al.*, 2000) which entrap the sperm and prevent further ascent. Other mechanical obstructions to the sperm at this particular location are provided by a vascular plexus in the submucosa of the oviductal wall, which can compress the oviductal lumen when engorged (Wrobel *et al.*, 1993) and the presence of a viscous mucus that can impede the progress of sperm (Suarez *et al.*, 1997). However, the actual entrapment of the sperm in the sperm reservoir at the uterotubal junction is probably not primarily caused by mechanical hindrance of the sperm progress (although this may be useful). Rather, there is strong evidence that this entrapment is the result of the sperm binding to the epithelium lining the oviduct (Suarez and Pacey, 2006). The binding of sperm to the oviductal epithelium is mediated by carbohydrate moieties, and in cattle the reservoir is formed by the binding of sperm to fucose-containing glycoconjugates on the surface of oviductal epithelial cells (Gwathmey *et al.*, 2003). This sperm-oviduct binding is reversible, since upon arrival of the ovulated oocyte in the ampulla, capacitated sperm are released from the endosalpingeal epithelium. This sperm release is probably the result of changes in heparin-binding sites of the sperm, as well as the induction of hyperactivated motility, which takes place in capacitated sperm, and not so much the result of changes in the binding sites of the oviductal epithelium (Suarez and Pacey, 2006). One could argue that if this were the case, then the influence of the female on sperm release would be minimal. However, there are indications that the process of capacitation is influenced by local and systemic ovarian control mechanisms, leading to changes in isthmus width, which means that the female is in control of the release of the sperm and thus coordinates the final maturation and the meeting of male and female gametes (Hunter and Rodriguez-Martinez., 2004). If this were true, hormonal imbalances which may be present in high producing dairy cattle would influence sperm transport/capacitation and hence impair fertilization. Until now there have been no arguments pleading for this line of reasoning, since no evidence has so far been reported that the numbers of sperm which are present in the sperm reservoir around the time of ovulation are reduced in high producing dairy cattle. There is only evidence for reduced fertilization rates in lactating dairy cows (but not in heifers) during the summer in hot countries due to heat stress (Sartori *et al.*, 2002). These authors flushed the embryos in different groups of cattle at day 5 and found a high number of accessory sperm on embryos and unfertilized oocytes, which indicated that sufficient numbers of sperm had reached the oocyte. Even significantly greater numbers of accessory sperm were found in lactating cows than in heifers, which was surprising in light of the larger reproductive tract in cows. This led the authors to conclude that the presence of accessory sperm in most unfertilized oocytes in lactating cattle exposed to heat stress was consistent with fertilization failure being due to an oocyte problem (Sartori *et al.*, 2002).

In conclusion, these data show that an impaired sperm transport and a decreased number of sperm in the sperm reservoir may not be the primary cause of infertility in lactating dairy cows in Belgium. Care should be taken to minimize male fertility problems that could produce fertilization failure in dairy herds, such as low quality semen, inappropriate AI technique or inappropriate timing

of AI. We can safely assume that lactating dairy cattle in Belgium are being inseminated by experienced inseminators with high quality semen, since we found in a recent field trial that a reduction of the insemination dose from 12 million to 2 million spermatozoa did not decrease conception rates either in heifers or cows, although the heifers had significantly higher pregnancy rates (Verberckmoes *et al.*, 2005). Moreover, the stringent selection criteria for Holstein sires preclude almost completely the use of suboptimal semen in dairy cattle.

EMBRYO QUALITY

In the paragraphs above we have tried to show that the conditions under which an oocyte may develop in high producing dairy cattle are not optimal. As far as sperm capacitation is concerned, no evidence so far leads us to believe that this part of the reproductive process may be impaired in dairy cattle. However, the next step following successful fertilization is the growth and development of a healthy embryo. It is clear from data in the literature that much higher conception rates are prevailing in Holstein heifers (about 60-75 %) than in lactating cows (25-40 %) (reviewed by Wiltbank *et al.*, 2006). A very useful *in vivo* approach to determine the cause of conception failure has been to flush embryos at given stages after breeding to determine fertilization failure and timing of embryonic mortality. Only a limited number of studies using this approach with normal ovulating, high-producing, lactating dairy cows are available, and they show that the fertilization rates are not really affected by lactation (but are decreased in dairy cattle exposed to heat stress) (Wiebold, 1988; Ryan *et al.*, 1993; Sartori *et al.*, 2002). Moreover, the number of viable embryos was significantly lower for lactating dairy cattle compared to non-lactating cattle. In the latter study of Sartori and coworkers (2002), embryo quality was assessed by means of an embryo grading system as described by Ahmad *et al.* (1995), and the number of blastomeres in each embryo was counted by means of Hoechst staining. Both this non-invasive and the invasive approach showed decreased embryo quality in lactating cattle.

Other field trials have failed to demonstrate the relevance of embryo quality for decreased fertility in dairy cattle. It seems very difficult to prove the hypothesis that high producing dairy cows have decreased early embryonic development on the basis of reviewing commercial embryo transfer results. In a recent summary of Holstein superovulatory results spanning a 20-year period in the USA, no indication was found of a decline in the mean number of transferable embryos resulting from superstimulation (Hasler *et al.*, 2006), and no correlation between embryo production following superstimulation and milk production was found. Whether there was a decline in pregnancy rate after the transfer of these embryos was even more difficult to ascertain. Although no evidence was available that the pregnancy rates had changed over the course of the study, the authors admitted that it was difficult to get hold of pregnancy data, since veterinary practitioners are no longer paid (as they used to be) on the basis of the number of pregnant recipients (determined several months after ET by rectal palpation) but rather on the basis of the number of embryos transferred (Hasler *et al.*, 2006). We experienced the same problem (i.e. no access to pregnancy data) in a field study on embryo quality in superovulated lactating dairy cows, non-lactating dairy heifers and beef cows (Leroy *et al.*, 2005b). In this field study, we discovered a significant difference in embryo morphology between lactating dairy cows and non-lactating dairy heifers and beef cows (see Leroy *et al.*, 2006). Another change in commercial embryo transfer is the high percentage of embryos which are now frozen following collection, making it even more difficult to get a meaningful representation of pregnancy rates after ET. Finally, pregnancy results after ET are predominantly determined by the characteristics of the recipient (synchronization, age, nutrition) (McMillan, 1998).

To fully explore the role of embryo quality in the lower conception rate of lactating dairy cows, specific studies are necessary to relate abnormal reproductive parameters with the level of milk production or with the metabolic stress experienced by dairy cattle. Such specific studies might aim to look at the embryo quality in lactating dairy cows on the basis of subjective morphological assessment of the embryos. Assessment of embryo morphology can indeed reveal a lot on embryo quality (Van Soom *et al.*, 2003), but it may be worthwhile to complement this method of embryo grading by means of invasive techniques (Van Soom and Boerjan, 2002) in order to detect which developmental problem causes these embryos to display aberrant morphology or developmental timing. Invasive techniques are not often used on embryos from commercial embryo transfer teams, since the embryo needs to be sacrificed to obtain the information wanted, and in most cases the embryo (which is per definition derived from a genetically superior dam and sire i.e. high producing dairy cattle) is needed for transfer to yield a calf.

In this respect it may be of benefit to take the *in vitro* embryo production process as a model for the high producing dairy cow. Bovine *in vitro* fertilization has in recent years expanded our knowledge on how external influences can affect embryo development and, even more importantly, can affect fetal development and eventual offspring.

Basically, *in vitro* produced cattle embryos are generated after rather indiscriminate selection of oocytes from

ovaries collected at slaughterhouses. Such oocytes are subjected to an in vitro maturation treatment for about 24 hours, and are subsequently fertilized with frozen-thawed sperm, which is selected over a density gradient to exclude viable from non-viable sperm. About 70 to 80% of fertilized oocytes start to cleave when cultured in adequate media and 20 to 40 % of them reach the transferable stage at 6, 7 or 8 days post insemination. The interesting thing about these *in vitro* produced (IVP) embryos is that:

- they are available on a daily or weekly basis (as required),
- they can be subjected to different culture conditions (which may mimic certain metabolic conditions in high producing dairy cattle),
- they can be evaluated at any given time after fertilization (which means that every developmental stage from immature oocyte to hatched blastocyst is accessible),
- and that they can easily be analyzed with invasive techniques, because their genetic value is very low to non-existent (since they are derived from culled cattle and experimental bulls).

Invasive techniques that are routinely applied on in vitro produced embryos and which may generate a great deal of information about *in vivo* produced embryos are listed in Table 2.

Especially noteworthy is the fact that embryos of high yielding dairy cattle are reminiscent of in vitro produced cattle embryos for two reasons: (1) they have a darker appearance, and (2) they have increased sensitivity to freezing (Geldhof, personal communication). These are precisely two characteristics of in vitro produced bovine embryos which are probably also a reflection of inadequate culture conditions: serum-containing culture conditions generate embryos with a dark appearance, which is probably caused by more lipid accumulation in the cytoplasm (Abe *et al.*, 2002; Leroy *et al.*, 2005). It has indeed been shown that culture in the presence of serum leads to more lipid-rich embryos. It is not immediately clear how this lipid increase happens: metabolic studies on ruminant embryos that have been cultured *in vitro* have shown that energy metabolism in such embryos is initially low and dependent on oxidative phosphorylation for the generation of ATP (Sinclair *et al.*, 2003). Pyruvate is a very important metabolic substrate for the embryo during this period, but much less is known about the metabolic fate of amino acids and lipids. Approximately 50% of the lipid fraction in the mature oocyte is in the form of triglyceride, much of which is oxidized during fertilization and the early cleavage stages. A very simple explanation for the finding that *in vitro* produced embryos are very dark and lipid-rich is that the media in which they are cultured are too rich for them, which leads their metabolism in an unhealthy direction. Culturing the embryos in serum-free media can indeed improve their ap-

Table 2. Examples of invasive methods that can be useful for evaluating embryo quality under specific *in vitro* culture conditions.

Embryo quality parameter to be evaluated	Technique	Reference
Allocation of cells to inner cell mass or trophectoderm	Differential staining	Van Soom *et al.*, 2002
Lipid accumulation in embryonic cells	Nile red	Leroy *et al.*, 2005c
Ultrastructure of cell organelles	TEM	Maddox-Hyttel and Boerjan, 2002
Detection of gene transcription	RT-PCR	Goossens *et al.*, 2005
Chromosomal abnormalities	FISH	Jakobsen *et al.*, 2006
Detection of apoptosis	TUNEL, Annexin-V staining, Caspase staining	Vandaele *et al.*, 2006

pearance and lead to less dark embryos: moreover, embryo culture in the absence of serum seems to normalize lamb birth weights (Thompson et al., 1995).

On the basis of this knowledge, we can clearly state that embryo viability, lipid content and energy metabolism can be influenced by environmental conditions. It is conceivable that this could also be the case in high producing dairy cows in which the uterine environment may be altered due to their changed metabolism (energy balance and typical highly nutritious diets). Further research should elucidate this.

CONCLUSION

When a sperm cell meets an oocyte under optimal circumstances, we can reasonably expect fertilization to occur. There is ample evidence that fertilization is taking place at normal levels in lactating dairy cattle, but the quality of the resulting embryo is probably impaired. This could either be caused by the direct effect of toxic metabolites on oocyte maturation and thus also on subsequent embryo development and/or by an indirect effect on the utero-oviductal environment. Which of these two explanations is the most likely, remains to be investigated. *In vitro* models of oocyte maturation and embryo development combined with invasive techniques of oocyte/embryo assessment may provide useful tools to shed light on this complicated area of reproductive physiology.

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