TYPICAL METABOLIC CHANGES IN HIGH PRODUCING DAIRY COWS EARLY POSTPARTUM AND THEIR CONSEQUENCES ON OOCYTE AND EMBRYO QUALITY

De typische metabole veranderingen bij hoogproductieve melkkoeien en de gevolgen voor de eicel- en embryokwaliteit

J.L.M.R. Leroy[1], T. Vanholder[2], G. Opsomer[1], A. Van Soom[1], P.E.J. Bols[3], P. Bossaert[1], A. de Kruif[1]

[1]Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine,
Ghent University, Salisburylaan 133, B-9820 Merelbeke
[2]Veterinary Centre Someren,
Slievenstraat 16, 5711 PK Someren, The Netherlands
[3]Laboratory for Veterinary Physiology, Department of Veterinary Sciences,
University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk

Jo.leroy@UGent.be

ABSTRACT

The negative energy balance (NEB) is characterized by typical biochemical changes such as high non-esterified fatty acid (NEFA), high β-hydroxybutyrate (BHB) and low glucose concentrations. The concentrations of these metabolites were extensively analyzed in the follicular fluid of high yielding dairy cows during NEB and were imitated in *in vitro* maturation models to investigate their effects on oocyte quality. The studies reviewed in this paper showed that the typical metabolic changes that occur during NEB are well reflected in the follicular fluid (FF) of the dominant follicle. However, the oocyte seems to be relatively isolated from excessively high NEFA or excessively low glucose concentrations in the blood. Nevertheless, the *in vitro* maturation models revealed that such metabolic changes in the FF associated with a NEB (high NEFA and low glucose concentrations) are indeed toxic for the oocyte, resulting in hampered oocyte maturation, increased apoptosis and necrosis in the cumulus cells, and jeopardized developmental competence of the resulting embryos. Only in moderately hypoglycemic maturation conditions did BHB have an additive toxic effect. These *in vitro* maturation models, based on *in vivo* observations and reviewed in this paper, suggest that a period of NEB may hamper the fertility of high yielding dairy cows through increased NEFA and decreased glucose concentrations in the FF, directly affecting oocyte quality. Finally, it was also demonstrated in our lab that the embryo quality of lactating high producing dairy cows is inferior compared to that of non-lactating dairy heifers or beef cows.

SAMENVATTING

De periode van de negatieve energiebalans (NEB) vroeg na het afkalven wordt gekenmerkt door heel typische metabole veranderingen, zoals hoge vrije vetzuren- (VVZ) en β-hydroxyboterzuur- (BHB) en lage glucoseconcentraties. Deze metabolieten werden uitvoerig bestudeerd in het follikelvocht van de dominante follikel. De studies die in dit artikel worden besproken, tonen aan dat de typische metabole wijzigingen tijdens de periode van NEB wel degelijk weerspiegeld worden in het follikelvocht van de dominante follikel. De eicel blijkt wel min of meer gevrijwaard te zijn van te hoge VVZ- en lage glucoseconcentraties. De concentraties gemeten *in vivo* werden nagebootst in *in vitro* maturatiemodellen en de resultaten tonen aan dat de hoge VVZ en lage glucoseconcentraties wel degelijk toxisch zijn voor de maturerende eicel. Alleen onder matige hypoglycemische omstandigheden bleek BHB een additief toxisch effect uit te oefenen. Al deze studieresultaten duiden aan dat een diepe NEB de fertiliteit negatief kan beïnvloeden door een rechtstreeks ongunstig effect op de eicel. Tenslotte werd in een veldstudie aangetoond dat lacterende hoogproductieve melkkoeien minder goede embryo's produceren in vergelijking met niet-lacterende melkveevaarzen en dikbilkoeien.

INTRODUCTION

Reproductive failure in high producing dairy cattle is a multifactorial problem. The pathogenesis of this subfertility is complex and especially the interactions between negative energy balance (NEB) early postpartum and the hypothalamus-pituitary-ovary-uterus axis have been studied thoroughly (Ducker *et al.*, 1985; Lucy, 2001; Butler, 2003). The disturbed endocrine signaling leads to a retarded resumption of ovarian cyclicity postpartum, which has been recognized as a major factor contributing to the reproductive failure that is often experienced by high yielding dairy cattle (Opsomer, 1998). However, attention has recently been shifting towards the ubiquitously reported disappointing conception rates (Bousquet *et al.*, 2004) and the remarkably high incidence of early embryonic mortality (Dunne *et al.*, 1999; Mann and Lamming, 2001; Bilodeau-Goeseels and Kastelic, 2003). Therefore, it is of crucial significance to concentrate on the quality of the oocyte and embryo proper in order to approach the problem of subfertility adequately (O'Callaghan and Boland, 1999). Are the intrinsic quality of the oocyte and the of embryo, which are the most essential factors for living offspring, compromised in modern high yielding dairy cows?

Recent studies have confirmed that the female gamete and the embryo are probably in danger (Kruip *et al.*, 1995; Kendrick *et al.*, 1999; Gwazdauskas *et al.*, 2000; Wiltbank *et al.*, 2001; Walters *et al.*, 2002). Snijders *et al.* (2000) studied the *in vitro* developmental competence of oocytes from dairy cows with either a high or a moderate genetic merit for milk production. Oocytes from high genetic merit cows resulted in significantly lower blastocyst yields *in vitro*, irrespective of milk production as such. This suggests possible adverse effects of the enforced genetic selection towards milk production on fertility.

Apart from oocyte quality, Wiltbank *et al.* (2001) demonstrated that non-lactating dairy cows yielded significantly more good quality embryos than lactating ones. Sartori *et al.* (2002) also focused on the quality of day 5 embryos at 2 to 3 months after calving. They found that embryos from lactating dairy cows were remarkably inferior compared to embryos from non-lactating cows or maiden heifers, and what is more, a high proportion of non-viable embryos were described in lactating cows (Sartori *et al.*, 2002). More studies are needed to get an informative and overall picture of average embryo quality in high yielding dairy cows.

It is obvious that reduced oocyte and/or embryo quality has been conclusively demonstrated in high yielding dairy cows. The comprehensive documentation on reduced conception rates and on the increased incidence of early embryonic mortality (Dunne *et al.*, 1999; Bousquet *et al.*, 2004) clearly indicates that the healthy growth and maturation of the female gamete and/or the normal development of the early embryo may be compromised. In the literature, many speculations and suggestions on the causes of these observations have been made, which are expressed in the following questions:

- ✓ Are the oocyte growth and maturation hampered well before ovulation due to biochemical alterations in the intrafollicular environment (O'Callaghan and Boland, 1999; Lozano *et al.*, 2003)?
- ✓ Has the microenvironment of the oviduct or uterus been changed due to dietary and metabolic changes in the modern dairy cow, creating a hostile environment for the early embryo (Elrod and Butler, 1993; McEvoy *et al.*, 1995; Kenny *et al.*, 2002)?
- ✓ Is something going wrong with the genetic information of modern dairy cow oocytes due to the consecutive years of rigorous genetic selection towards milk yield (Snijders *et al.*, 2000)?

In contrast with the extensive knowledge of the disturbed endocrine signaling and ovarian function, clear evidence concerning the impact of hampered oocyte and/or embryo quality on the final reproductive performance in high producing dairy cows is lacking. In the present article we will review the possible mechanisms, which have been studied in detail in our laboratory, by linking negative energy balance (NEB) to oocyte quality. Furthermore, in the event a successful fertilization takes place and an embryo is formed, it is not known whether the quality of this early life is impaired or not. Therefore, by means of a field trial, we compared the embryo quality between lactating high yielding dairy cows, non-lactating dairy heifers and beef cows.

FOLLICULAR FLUID, THE LINK BETWEEN BLOOD AND GAMETE

Linking NEB and oocyte quality is not an easy task. It is well known that NEB is characterized by some typical endocrine and biochemical changes in the blood of modern dairy cows (Herdt, 2000). Some

studies have already associated these metabolic changes in serum with oocyte quality without investigating the physiological connection between blood and oocyte: the follicle and the follicular fluid (Hashimoto *et al.*, 2000; De Wit *et al.*, 2001; Jorritsma *et al.*, 2004).

The follicle is an avascular 'compartment' filled with follicular fluid in which the oocyte undergoes the fine tuned process of oocyte growth, prematuration and final maturation (Bagavandoss *et al.*, 1983; Gosden *et al.*, 1988). The physiological basis of follicular fluid has been reviewed by Gosden *et al.* (1988). During the process of follicular growth, the physicochemical properties of the blood-follicle barrier change thoroughly, suggesting that the oocyte's environment undergoes compositional changes (Edwards, 1974; Wise, 1987; Gosden *et al.*, 1988). In addition, the active transport mechanisms through the follicular wall may also alter during follicular growth. Argov *et al.* (2004), for example, recently demonstrated that while lipoproteins are predominantly internalized by endocytosis in small follicles, this is not the case in large follicles in which circulating lipoproteins contribute their cholesterol esters by selective uptake and without internalization of the lipoprotein as such. Hence, to learn more about the rather unexplored biochemical world of the oocyte before ovulation, we analyzed the composition of follicular fluid originating from three differently sized follicles and compared it with the composition of serum from 30 dairy cows shortly postmortem (Leroy *et al*., 2004a). The data of this first experiment confirmed indeed that the follicular fluid composition changes as the follicle grows from small to large. Another important finding from this first experiment is the fact that the follicular fluid composition is correlated with the composition of the serum. The given correlations are 'static', however, and some words of clarification are needed. This means that when one cow has, for example, a higher glucose serum concentration compared to another cow, then this will also be the case for the glucose concentrations in the fluid of her follicles. So, strictly speaking, it is wrong to conclude from these data that when the glucose levels rise in the serum of a cow, this rise will be paralleled in the follicular fluid. For the confirmation of this particularly 'dynamic' correlation, we decided to explore the follicular fluid composition in living high yielding dairy cows by means of a repeated transvaginal follicle puncture.

Thus, in a second study (Leroy *et al.*, 2004b) we concentrated on compositional changes over time in the follicular fluid of high yielding dairy cows early postpartum. In this way we were able to compare these data with the already extensively studied biochemical alterations in blood during NEB (see above). Negative energy balance typically causes some obvious changes in serum such as high non-esterified fatty acids (NEFA) and β-hydroxybutyrate (β-OHB) concentrations or low glucose concentrations (Baird, 1982; Chilliard *et al.*, 1998; Duffield, 2000; Herdt, 2000). Urea concentrations can also be high due to an increased amino acid metabolism for gluconeogenesis or due to the intake of protein rich diets (Butler 1998; Sinclair *et al.*, 2000). Britt (1992) hypothesized that these features of the NEB can directly affect the follicle and the enclosed oocyte, leading to the ovulation of an inferior oocyte. This hypothesis is plausible since it is generally accepted that oocytes are highly vulnerable to any disruption in their environment (O'Callaghan and Boland, 1999), a point of view which has been more or less confirmed by others (Boland *et al.*, 2001; Armstrong *et al.*, 2001). We were convinced that the data of this second experiment were essential for introducing the first scientific evidence for this generally accepted 'Britt hypothesis', which is intended to answer the question: 'How does NEB directly influence the micro-environment which is most intimately linked with the oocyte?'(Britt, 1994).

An adapted ovum pick-up technique (Bols *et al.*, 1995) turned out to be a perfect method for collecting follicular fluid from the dominant follicle at 6 different points in time postpartum. Since we already knew from our previous research that follicular size can influence follicular fluid composition, it was important to aspirate similar sized follicles throughout the whole study. Because of the reduced approachability of the ovaries during the puerperium period, follicular fluid was only collected from day 14 postpartum onwards.

By analogy with the first experiment, 'static' correlations per point in time postpartum between serum and follicular fluid composition were calculated, and they confirmed the results of the first study. Especially for glucose, β-OHB, urea and total cholesterol, good correlations were found. Based on the results of the repeated measurement design (dynamic correlations) (Leroy *et al.*, 2004b), we can say now that those typical postpartum serum fluctuations are more or less reflected in the follicular fluid of the dominant follicle. For urea and β-OHB, no concentration differences between serum and follicular fluid could be detected. It is important to mention, though, that the follicle is able to maintain higher glucose and lower NEFA concentrations compared to serum. Or, in other words, it can be suggested that the oocyte is isolated (or even

protected?) from excessively low glucose or excessively high NEFA concentrations present in the blood.

In spite of the follicle's buffering capacities, glucose concentrations do decrease and NEFA concentrations significantly rise in follicular fluid during NEB. Also Jorritsma *et al.* (2003) and Comin *et al.* (2002) described a NEFA rise in follicular fluid concentrations parallel with an increase in the serum concentrations due to an acute dietary restriction. No concentration gradients between serum and follicular fluid have been mentioned, however. It is only recently that Hammon *et al.* (2005) confirmed our findings concerning follicular urea concentrations in high producing dairy cows early postpartum. There is now enough evidence to conclude categorically that the growing and maturing oocyte is directly exposed to the typical biochemical changes which occur in high yielding dairy cows early postpartum. It has furthermore been proven that high urea concentrations can be toxic for oocytes during maturation (Ocon and Hansen, 2003; Iwata *et al.*, 2005), probably through an inhibition of the polymerization of tubulin into microtubules (De Wit *et al.*, 2001). The same is true for the observed low glucose concentrations. Adequate glucose supplies are necessary to support normal cumulus expansion and nuclear maturation (Krisher and Bavister, 1998; Sutton-McDowall *et al.*, 2004). Similarly, high NEFA and β-OHB concentrations are probably harmful for the oocyte's developmental competence, but this has, to our knowledge, never been substantiated.

Therefore we subsequently concentrated on possible adverse effects of high NEFA or β-OHB concentrations, as have been described for urea and glucose (Chapter 5). As in other studies, we were also obliged to use *in vitro* maturation models to get answers to these questions. Since *in vitro* media are only an approach to the real *in vivo* conditions, the results should always be interpreted with caution!

NEGATIVE ENERGY BALANCE AND THE DIRECT CONSEQUENCES FOR OOCYTE QUALITY: AN *IN VITRO* MODEL

First of all, attention was paid to the NEFA fraction of follicular fluid (Leroy *et al.,* 2005a). Since NEFA are a family of all kinds of fatty acids, new and specialized measurements on the follicular fluid of high yielding dairy cows during NEB were needed. Not only the absolute NEFA concentration but also the NEFA composition needed to be analyzed by means of a combined thin layer and gas chromatography (Folch *et al.*, 1957). The results surprisingly revealed that not only the NEFA concentration (see above) but also the NEFA composition significantly differs between serum and follicular fluid (Leroy *et al*., 2005a). Differences in albumin concentration (on which NEFA are predominantly bound) between the two compartments could not be found and thus did not offer any clue for explaining the observed differences in NEFA concentration and composition. Additionally, the described dynamic interchange of NEFA between serum and follicular fluid (Moallem *et al.*, 1999) is also not really in line with our findings. It was a study of Chung *et al.* (1995) that reported a possibly useful clarification for our study results. In the presence of high NEFA levels, a substantial portion of the NEFA in serum is partitioned to low density lipoproteins (LDL). Especially the saturated fatty acids are bound on LDL, while the unsaturated ones are preferably bound on albumin (Chung *et al.*, 1995). Because LDLs are absent in FF, these findings could account for the differences in concentration and composition of NEFA in FF compared to serum early postpartum (Wehrman *et al.*, 1991). Further research to confirm this hypothesis is desirable however.

Whatever the mechanisms are, we now know the concentrations of the three most abundant fatty acids present in follicular fluid during NEB: oleic, palmitic and stearic acid. These concentrations were applied during an *in vitro* maturation model to evaluate their effect on oocyte quality (Leroy *et al.*, 2005a). In our tests, oleic acid had no discernable effect on the oocyte. However, exposing oocytes to palmitic and stearic acid at concentrations comparable to those assessed *in vivo*, resulted in reduced maturation leading to disappointing fertilization and cleavage rates. Also cumulus expansion was hampered. In these cumulus cells, a significantly higher rate of apoptosis and even necrosis could be detected after 24 hours of exposure to high stearic or palmitic concentrations. Similar toxic effects on bovine or human granulosa cells *in vitro* have been shown in other studies (Mu *et al.*, 2001; Jorritsma *et al.*, 2004; Vanholder *et al.*, 2005). Optimal granulosa and cumulus cell functioning is indispensable for oocyte maturation because these cells are responsible for endocrine and paracrine signaling (Bilodeau-Goeseels and Panich, 2002; Tanghe *et al.*, 2002). Therefore it is most likely that the toxic effect of NEFA on oocyte quality is mainly an indirect effect.

In contrast with our results, Jorritsma *et al.* (2004) did find detrimental effects of oleic acid. In their study, however, oleic acid was bound on albumin and was added in supraphysiological concentrations to an un-

defined *in vitro* maturation medium (addition of fetal calf serum, which is a source of fatty acids). Moreover, it is unclear whether these adverse effects were caused by the addition of BSA itself or by oleic acid. Homa and Brown (1992) showed that albumin bound linoleic acid in IVM medium inhibited germinal vesicle breakdown in denuded oocytes. Similar toxic effects of NEFA have also been described for Leydig cells, muscle cells and pancreatic β-cells, and it is especially the induction of apoptosis and/or insulin resistance and changes in membrane properties that have been suggested as potential mechanisms for explaining the observed toxic effects (Shimabukuro *et al.*, 1998; Maedler *et al.*, 2001; Hirabara *et al.*, 2003; Lu *et al.*, 2003; Jorritsma *et al.*, 2004).

The results of this study are not only important in relation to the subfertility issue in modern dairy cows, but may also be a valuable model for human research. Obesity and diabetes are characterized by increased concentrations of NEFA due to high adipose sensitivity for lipolytic triggers (Herdt, 2000; Cnop *et al.*, 2001). Our data may suggest that the frequently reported fertility disorders in obese or diabetic women (Pasquali *et al.*, 2003) are not only due to the toxic effects of NEFA on granulosa cells, which mainly lead to amenorrhea (Mu *et al.*, 2001), but may also originate from the direct harmful effects on the cumulus-oocyte-complex. The latter could explain the disappointing IVF or ICSI results and the higher risk for early pregnancy loss in obese women, as has been documented by Fedorcsak *et al.* (2000; 2004) and Pasquali *et al.* (2003). Further research should confirm the appropriateness of this bovine model in human medicine.

Not only high NEFA but also elevated ketone concentrations are a distinctive characteristic of NEB (Sato *et al.*, 1999). High ketone concentrations mostly go together with hypoglycemia (Herdt, 2000). In a second IVM model (Leroy *et al.*, 2006), we therefore investigated the effect of combined high β-OHB and low glucose concentrations derived from measurements in follicular fluid of dairy cows during NEB. The main conclusion of this study was that the *in vitro* model imitating subclinical ketosis had no effect on the oocyte's developmental capacity *in vitro*. Clinical ketosis, however, turned out to be harmful to oocyte quality *in vitro*: this was due to the low glucose concentrations rather than to the effect of high β-OHB concentrations. Thus the toxicity of β-OHB as has been described for polymorphonuclear cells and macrophages (Hoeben *et al.*, 1997; Sartorelli *et al.*, 2000) could not be confirmed for oocytes. On the other hand, we can conclude with a high degree of certainty that inadequate glucose supplies may compromise oocyte developmental competence, a conclusion which is in line with other studies (Krisher and Bavister, 1998; Cetica *et al.*, 2002; Sutton-McDowall *et al.*, 2004).

When interpreting these *in vitro* results and translating them in terms of subfertility in high producing dairy cows, some prudence is warranted. In our study we hypothesized that elevated NEFA or β-OHB concentrations together with low glucose concentrations may contribute to reducing fertility in high yielding dairy cows by exerting detrimental effects on oocyte developmental competence. Our findings are more or less in line with the hypothesis of Britt (1994), who hypothesized that a follicle grown during the period of NEB early postpartum could be affected by the unfavorable metabolic changes and may contain a developmentally incompetent oocyte; after a growing and maturation phase of several weeks, this inferior oocyte will be ovulated at the moment of the first insemination (Lucy, 2003). This hypothesis has more or less been confirmed in recent *in vivo* studies (Gwazdauskas *et al.*, 2000; Snijders *et al.*, 2000; Sartori *et al.*, 2002). It is important, however, to mention that the combined *in vitro* and *in vivo* model described above was not entirely appropriate for investigating the above-described carry-over effect on oocyte quality as hypothesized by Britt (1994). Our results only documented the follicular fluid composition in the dominant follicle during the NEB mimicked *in vitro*. Quiescent follicles embedding the oocytes of interest, however, provide a much poorer isolation of the oocyte from the extrafollicular environment and blood serum. As a consequence, such oocytes are probably exposed to even higher NEFA concentrations (Zamboni, 1975). Another possibility is that oocytes of primordial follicles are completely insensitive to all these metabolic disruptions. Moreover, in the present study the cumulus oocyte complexes were exposed to elevated NEFA or β-OHB and low glucose concentrations for only 24 h, whereas *in vivo* the oocytes are exposed to such concentrations for several days or even weeks. In the ideal model, primordial follicles should be cultivated in high NEFA conditions for several weeks. However, such long-term cultures of primordial follicles still have major drawbacks and growing bovine primordial follicles up to the preovulatory stage has so far proven to be impossible (Gutierrez *et al.*, 2000), although successes have been obtained in mice with prolonged primordial and preantral follicle culture, leading to *in vitro* ovulation and, after fertilization, the birth of pups (Cortvrindt and Smitz, 2001). Nevertheless, we

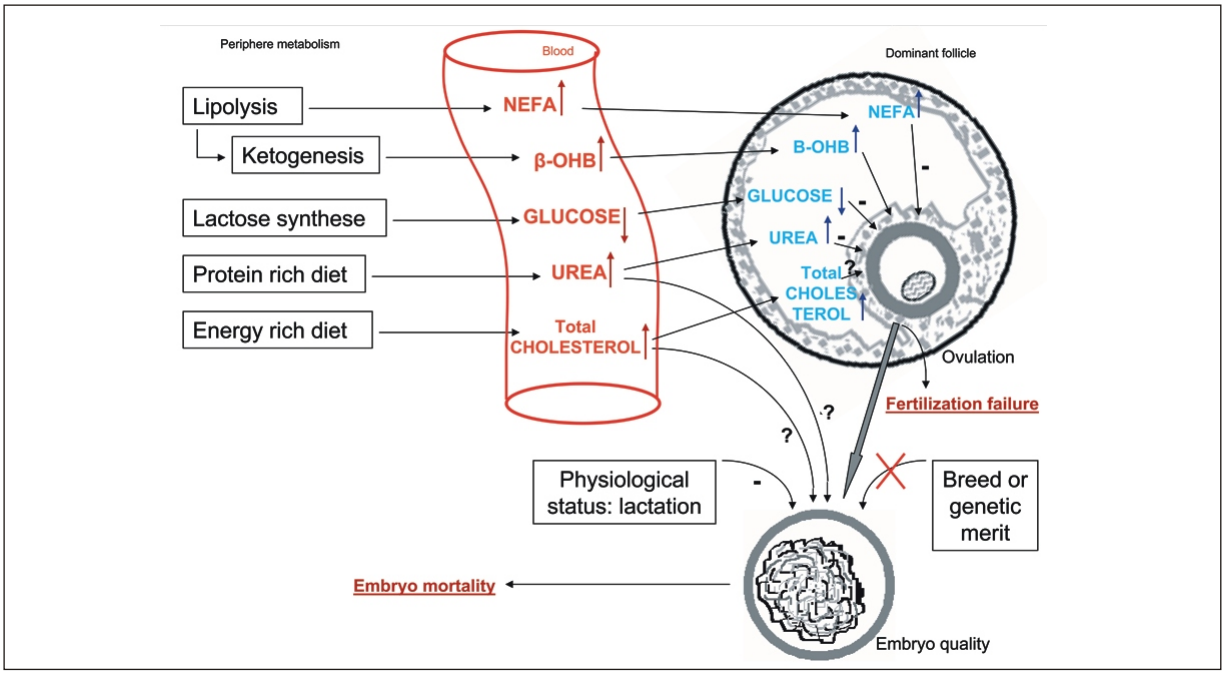

Figure 1. Diagrammatic representation of possible mechanisms by which embryo quality can be impaired in high yielding dairy cows.

do believe that the model used in the present study revealed for the first time possible toxic effects of high follicular fluid NEFA and low glucose concentrations on the developmental competence of bovine oocytes *in vitro*.

BACK TO THE FIELD: A CLOSER LOOK AT EMBRYO QUALITY

Until now we have predominantly focused on oocyte quality in relation to NEB. From the above it can be suggested that the oocyte is vulnerable to some of the metabolic alterations associated with this NEB. At least *in vitro*, obvious adverse effects on oocyte quality were observed. Logically, the next step would be to investigate the consequences for embryo quality. As has been suggested by Rizos *et al.* (2002), the conditions prior to fertilization are determinant for embryo yield, while the embryo culture environment is crucial for embryo quality. On the basis of that theory, the toxic effects of high NEFA and low glucose concentrations during oocyte maturation that have been demonstrated in earlier work (Leroy *et al.*, 2005a; Leroy *et al.*, 2006) will mainly lead to low fertilization (i.e. conception) rates in high producing dairy cows. And on the basis of the reports of Bousquet *et al.* (2004) this does seem to be the case. However, not only the environment of the oocyte but also that of the embryo is said to be crucial for final fertility. As suggested by Kenny *et al.* (2002), Elrod and Butler (1993) and McEvoy *et al.* (1995), high energy and/or high protein diets can alter the microenvironment of the embryo in the oviduct and uterus. Such changes are expected to be pernicious for embryo quality (Rizos *et al.*, 2002), which has been shown experimentally by Wrenzycki *et al.* (2000) in heifers. However, it has never been demonstrated whether this is also the case in high yielding dairy cows. Therefore, we set up a field trial to gain more insight into the embryo quality of high producing dairy cows in comparison with non-lactating dairy heifers and beef cows. In this way we were able to investigate the effects of producing both milk and breed (or genetic background) (Leroy *et al.*, 2005b). Briefly, lactating dairy cows clearly displayed inferior embryo quality as assessed by morphological evaluation, compared to dairy heifers or beef cows. Furthermore, we were able to demonstrate by means of a multivariable regression model that the fact of producing milk or not producing milk was significantly associated with embryo quality. It is also important to mention that no differences were found in fertilization rate or in the number of transferable embryos per embryo collection.

Since the embryos of the lactating dairy cows were on average collected around day 230 postpartum, it is very unlikely that a carryover effect of the NEB, as has been hypothesized by Britt (1992), is responsible for this observation. This could have been the case

when embryo collection was performed on average around two to three months after calving, as was done by Sartori *et al.* (2002), who also found an obvious difference in embryo quality between lactating dairy cows and maiden heifers. In contrast with our results, Sartori and coworkers (2002) reported not only inferior embryo quality but also a lower fertilization rate expressed as a higher proportion of unfertilized oocytes present in the uterine flushing of lactating dairy cows. Sartori *et al.* (2002) probably described the adverse influences of a carryover effect of NEB on oocyte quality (reduced fertilization rates), combined with the possible negative effects of lactation, management or diet on the microenvironment of the oviduct or uterus (reduced embryo quality), as we found in our field trial. By way of summary, all suggested mechanisms that could potentially hamper embryo quality are diagrammatically represented in Figure 1.

Further research should reveal the exact mechanism through which embryo quality is hampered in lactating dairy cows:

- ✓ Some of the physiological adaptations associated with milk production may have adverse effects on embryo quality. Two of the typical features of high milk production are high total cholesterol and low triglyceride concentrations in the blood (Varman and Schultz, 1968; Blum *et al.*, 1983), both of which were also found in our study. However, no direct associations of these parameters with embryo quality have as yet been found.

- ✓ As stated above, the typical milk stimulating rations high in energy and protein have been linked with reduced embryo quality (McEvoy *et al.*, 1997; Yaakub *et al.*, 1999). This will be discussed more extensively in future volumes of the Flemish Veterinary Journal.

One of the major morphological embryo characteristics we evaluated was color. We were able to confirm with our new lipid evaluation technique (Leroy *et al.*, 2005c) that embryo color is correlated with lipid content, as has previously been suggested by others (Sata *et al.*, 1999; Abe and Hoshi, 2003). Lactating dairy cow embryos were generally dark and contained as much lipids as *in vitro* produced embryos, which are known to accumulate excessive amounts of lipids (Abe *et al.*, 1999). This has never been shown before. Such a high lipid content has obviously been linked with impaired embryo quality (Reis *et al.*, 2003; Rizos *et al.*, 2003). The underlying mechanism linking milk production or nutrition with embryo color is not known and is a matter for further research.

PERSPECTIVES FOR FUTURE RESEARCH AND SOME FOOD FOR THOUGHT

As has been mentioned above, future research should teach us more about the interactions between the blood, the micro-environment in the oviduct or uterus and the embryo metabolism. Furthermore, several studies have indicated that NEB is also associated with depressed immunity during the first weeks postpartum, thus leading to an increased susceptibility to infectious diseases such as mastitis and metritis (Hoeben *et al.*, 2000; Lacetera *et al.*, 2005). Bearing this knowledge in mind, it is important to consider not only a direct link between NEB and fertility, as has been discussed extensively in this thesis, but that reproductive functions are also affected indirectly by the increased incidence of infectious diseases. Mastitis, for example, which – together with low fertility – is a major reason for the culling of dairy cows, has been proven to be directly linked to the retarded onset of ovarian activity postpartum (Loeffler *et al.*, 1999; Rajala-Schultz and Gröhn, 2001; Huszenicza *et al.*, 2005). Whether infectious diseases can affect the oocyte and/or the embryo in a direct way is poorly studied and certainly needs further research (Hansen *et al.*, 2004). In addition, environmental pollution has been associated with direct harmful effects on oocyte quality through the generation of endocrine disrupters (Brevini *et al.*, 2005).

Is there still such a need for high producing dairy cows? Yield maximization per animal is indeed preferable from an economical and environmental point of view. However, only outstanding herd management can guarantee the animal welfare in such high producing cows. But even then, the pressure on these animals remains high since they are rapidly culled for reasons such as reduced fertility, metabolic disorders and infectious diseases. The present article reveals that even the oocyte and the embryo may suffer from this high productivity.

CONCLUSIONS

It can be stated categorically that the typical biochemical serum changes during the NEB early postpartum are well reflected in the follicular fluid of the dominant follicle exposing the granulosa cells and the maturing oocyte. *In vitro* maturation models revealed that NEB associated NEFA and glucose concentrations

are indeed toxic for the oocyte, resulting in hampered oocyte maturation and less developmental competence.

Even when the period of NEB was over and when the carryover effects of the NEB were no longer present, high yielding dairy cows produced significantly inferior embryos in comparison with dairy heifers and beef cows. With a newly developed lipid evaluation technique, we were able to demonstrate that high producing dairy cow embryos contained up to 45% more lipids compared to the embryos of non-lactating animals. These findings imply that it is not the genetic merit for milk production or breed that has an impact on embryo quality, but rather that all kinds of factors associated with milk production as such (metabolism, nutrition, management) induce hostile conditions that prevent optimal embryo development. Further research is needed to learn more how milk production and the nutrition of the dairy cow can influence embryo health and metabolism by altering its environment in the oviduct and uterus.

We now have evidence that the occurrence of a NEB has harmful consequences for the quality of the female gamete, and that the fact that a cow is producing great quantities of milk (physiological mechanism sustaining milk secretion, nutrition, and management) is detrimental for normal embryo quality.

Yes, oocytes and embryos in high producing dairy cows really are in danger!

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