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Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum

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Abstract

Characteristics of the intrafollicular environment to which the preovulatory oocyte is exposed may be one of the major factors determining subsequent fertility. The aim of our study was to examine to what extent metabolic changes that occur in early post partum high-yielding dairy cows are reflected in the follicular fluid (FF) of the dominant follicle (>8 mm). Nine blood samples were taken per cow from nine high-yielding dairy cows between 7 days before and 46 days after parturition. From Day 14 post partum on and together with blood sampling, FF samples of the largest follicle were collected from the same cows by means of transvaginal follicle aspiration. Serum and FF samples were analyzed using commercial clinical and photometric chemistry assays for glucose, β-hydroxybutyrate (β-OHB), urea, total protein (TP), triglycerides (TG), non-esterified fatty acids (NEFA) and total cholesterol (TC). All cows lost body condition during the experimental period (0.94 ± 0.09 points) illustrating a negative energy balance during the experimental period. In FF, glucose concentrations were significantly higher and the TP, TG, NEFA and TC concentrations were significantly lower than in serum ($P < 0.05$). The concentrations of glucose, β-OHB, urea and TC in serum and in FF changed significantly over time ($P < 0.05$). Throughout the study, changes of all metabolites in serum were reflected by similar changes in FF. Especially for glucose, β-OHB and urea the correlations were remarkably high. The results from the present study confirm that the typical metabolic adaptations which can be found in serum of high-yielding dairy cows shortly post partum,

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are reflected in follicular fluid and, therefore, may affect the quality of both the oocyte and the granulosa cells.

Keywords: Dairy cow; High producing; Fertility; Follicular fluid; Metabolites

1. Introduction

Over the past decades there has been a substantial decline in the reproductive performance of high-producing dairy cows [1,2]. The negative energy balance (NEB) early post partum and the intake of a high-protein and high-energy diet, are known to cause hormonal and biochemical changes in these cows [3–7]. Physiological adaptations during the onset of lactation, such as hypoglycemia, ketonemia, uremia, increased levels of non-esterified fatty acids and subsequent lipid accumulation in the liver, can become pathological and hence may interfere with reproductive performance [8–12]. However, it is not completely clear how these biochemical changes are able to influence reproductive outcome. O'Callaghan and Boland [3] suggested that the decline in fertility is mainly a problem of inferior oocyte and embryo quality. Altered concentrations of gonadotropins, steroids and growth factors in follicular fluid (FF) have already been linked with changes in oocyte quality [13–15]. Furthermore, diets high in energy and protein, which are typically supplied to high-yielding cows, are known to alter oocyte and subsequent embryo quality probably through a changed composition of the follicular and/or fallopian tubal fluid [3,4,16–18].

A new approach to investigate the contribution of these metabolic adaptations to the pathogenesis of reduced fertility is to mimic biochemical changes in an in vitro model to study the possible effects on in vitro granulosa cell function [19] or on oocyte maturation, fertilization and subsequent embryo yield [20]. Such studies already showed that parameters, such as glucose, urea and β-hydroxybutyrate (β-OHB) may influence the competence of bovine oocytes to mature and, following fertilization, to develop to the blastocyst stage [16,20–23].

However, despite all these interesting data one important step has not been investigated yet. Little is known about the implications of post partum biochemical serum changes in the composition of the FF, embedding the granulosa cells and supporting the oocyte to undergo the fine tuned processes of growth, pre- and final maturation. In a previous study on dairy cows post mortem, we demonstrated that the oocyte and the granulosa cells grow and mature in a changing biochemical environment from small to large follicles. The biochemical composition of the FF was well correlated with the serum composition [24]. However, these findings needed to be confirmed in living, high-yielding dairy cows which were subjected to repeated sampling of serum and FF during the first weeks after calving.

Therefore, the objectives of the present study were (1) to investigate if the metabolite concentrations in serum and FF are significantly different and (2) to assess to what extent metabolic changes that occur in high-yielding dairy cows early post partum are reflected in the FF. Concentrations of glucose, β-OHB, urea, total protein (TP), triglycerides (TG), non-esterified fatty acids (NEFA) and total cholesterol (TC) were examined because of their importance in the metabolism of dairy cows.

2. Materials and methods

2.1. Animals

Nine healthy multiparous Holstein–Friesian cows were used in this study. All experimental work was performed at the research dairy farm of the University of Ghent (Biocentrum Agri-Vet, Melle, Belgium) following protocol approval by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University). Cows were milked on average 2.2 times a day by means of an automated voluntary milking system. On the farm, the average milk yield per cow was 9200 kg milk (3.90% fat and 3.45% protein) during 305 days of lactation.

After an average dry period of 55 ± 12 days in which the animals were fed corn silage, straw ad libitum, dry cow minerals, magnesium and soybean meal, all cows calved normally between September 2002 and April 2003. During the experimental period (first 50 days of lactation), all cows were housed in a loose stable with cubicles and were fed according to their requirements for maintenance and milk production. The ration consisted of high-quality roughages (corn silage and grass silage, sugar beet pulp), soybean meal and concentrates. Propylene glycol (500 ml daily) was routinely given as an oral drench to all cows during the first 3 days of lactation. One animal, that suffered from retained placenta, was treated once i.u. with tetracycline (4 g) at 24 h post partum. The fetal membranes have been removed the day after. One other animal suffered from a mild mastitis in one quarter. After an intramammary treatment with antibiotics, the animal was cured within 3 days, well before the first ovarian puncture. During the experimental period, daily milk yield ($\pm$S.E.M.) of the selected cows averaged 38.2 ± 2.7 kg per cow, ranging from 30.1 to 53.5 kg. Body condition scores (BCS), based on the notation of Edmondson et al. [25], were recorded by the same experienced person using a score on a scale of 1–5 (with 0.25 increments).

2.2. Blood sampling

Blood samples were collected from each animal 7 days prior to the expected calving date and at Days 0, 11, 14, 20, 26, 33, 40 and 46 post partum. Blood was sampled from the jugular vein into two unheparinized, silicone-coated tubes (Venoject®, Autosep®, Gel + Clot. Act.; Terumo Europe N.V., Leuven, Belgium) and in one tube with sodium fluoride (NaF) (Venoject®, Terumo Europe N.V.). Samples were taken between 1.00 p.m. and 3.00 p.m., 2 h after automated milking at the latest and before any rectal examination or ultrasound transvaginal aspiration. The coagulated blood samples and the blood samples on NaF were centrifuged ($1400 \times g$, 30 min) within 1.5 h after collection and the serum or plasma was collected.

2.3. Ultrasound examination and follicular fluid sampling

On Day 11 post partum all animals showed normal uterine involution and follicular growth on one or both ovaries upon ultrasound examination. On Days 14, 20, 26, 33, 40 and 46 post partum (experimental sessions), dominant follicles with a diameter greater than

0.8 cm were subjected to ultrasound guided transvaginal aspiration as described by Bols et al. [26]. Briefly, the rectum was emptied and the perineum and external genitalia were cleansed carefully. Cows received epidural anaesthesia (5 ml Procain HCl 4% with adrenalin, Eurovet N.V., Heusden-Zolder, Belgium) to prevent them from straining. An OPU device, equipped with a 5.0 MHz mechanical multi-angle probe transducer (Esaote/Pie Medical N.V., Maastricht, The Netherlands) and a needle guidance system (Pie Medical) was inserted vaginally and both ovaries were visualized through rectal manipulation. Before aspiration, the number of different sized follicles (<4, 4–8 and >8 mm) was recorded per ovary. Subsequently, follicles were punctured and the FF was aspirated by a second operator, following positioning along the biopsy line. Hereto, the needle (TERUMO NEOLUS 21G × 2 in., 0.8 × 50, Leuven, Belgium) was attached by means of a stainless steal connector to an extra thin silicon tube (inner diameter: 0.034 in.; Silclear TM Tubing, Multi Purpose Medical Grade Silicone Tubing, Degania Silicone/Israel) and a 5 ml syringe (Plastipak^{TM}, Madrid, Spain) was used to aspirate the FF from the punctured follicle. The largest and the second largest follicle (if present) with a diameter greater than 8 mm were aspirated. Attention was paid to prevent blood contamination. Follicular fluid samples with obvious blood contamination were omitted from further processing. The collected FF was cooled immediately (4 °C). Subsequently, FF samples were centrifuged (10,000 × *g*, 10 min) and the supernatant was collected for analysis. Sample preparation was completed within 3 h after each session. Blood and FF samples were snap-frozen in CO_2 ice (−65 °C) and stored at −22 °C until biochemical assay.

2.4. Hormone analyses

To identify possible atresia of the punctured follicles, a progesterone (P4) and estradiol-17β (E) analysis was carried out on each FF sample. Follicular fluid with a ratio E/P4 < 1 was considered to originate from an obviously atretic follicle and was omitted for biochemical analysis [27–29]. Progesterone was extracted with petroleum ether from 20 μl of FF that was diluted three times. Estradiol was extracted with diethyl ether from 20 μl of FF that was diluted 100 times. Estradiol-17β and progesterone concentrations were assessed through a radioimmunoassay (RIA), as described earlier [30]. The detection limit for E was 5 pg and the intra- and inter-assay coefficients of variation were 5.75 and 8.30%, respectively. The RIA for P4 had a detection limit of 5 pg and intra- and inter-assay coefficients of variation of 7.05 and 8.75%, respectively.

2.5. Biochemical analyses

In each sample, the concentrations of glucose, β-OHB, urea, TP, TG, NEFA and TC were measured. All analyses were performed at the Department of Clinical Chemistry, University Hospital, Ghent, Belgium. The determination of metabolite levels in FF and blood serum was done using wet chemistry techniques on two clinical chemistry automated analyzers (Modular P and Hitachi 911, Roche Diagnostics). Measurements of glucose, urea, TP, TG and TC were performed using commercial photometric assays (Roche Diagnostics GmbH, Mannheim, Germany). Commercial kits were also used for the measurement of β-OHB (Sigma Diagnostics Inc., St. Louis, USA) and NEFA (Wako

Chemicals GmbH, Neuss, Germany). All measurements were carried out according to the manufacturer's instructions. The intra- and inter-assay coefficients of variation for all analyses were below 5%.

2.6. Statistical analyses

All data are presented as means ± S.E.M. Only the data collected from Day 14 post partum onwards (availability of both serum and FF samples) were used in the statistical model. However, to have an overall view on serological changes pre- and post partum, the serum concentrations before Day 14 post partum are presented in the figures. Since data were correlated (repeated measurements in the same animal) a linear mixed effects model with cow as random factor (S-Plus 2000, Cambridge, USA) was used (1) to investigate if the metabolite concentrations were significantly different in the serum compared to FF (effect of compartment), (2) to evaluate if the metabolite concentrations changed significantly over time (effect of days post partum) and (3) to estimate to what extent the changes in serum and FF metabolite concentrations were parallel during the test period (interactions compartment × time post partum). A non-significant compartment × time interaction indicates that the concentrations of the metabolite of interest changes similarly over time in both compartments. The data for NEFA and TC concentrations were log-transformed for normality reasons. Normal correlations (Pearson) were calculated between serum and FF levels at each moment post partum (SPSS 11.0 for Windows, Chicago, IL, USA). A paired samples *t*-test was used to compare milk yield and BCS at the onset and at the end of the experimental period (SPSS 11.0 for Windows). Values of $P < 0.05$ were considered statistically significant.

3. Results

From 7 days prior to the expected parturition (varying between 11 and 3 days prior to the real day of parturition) up to 46 days post partum, all cows showed a significant loss in BCS (an average BC loss of 0.94 ± 0.09 points) ($P < 0.05$) (Fig. 1). From Day 11 up to Day 46 post partum, the average daily milk yield increased with 7.2 kg, from 35.7 ± 2.3 kg to 42.9 ± 3.5 kg. An average of 1.2 ± 0.1 follicles were punctured per session per cow and a total amount of 1.62 ± 0.14 ml FF was aspirated. Due to atresia, based on the E/P4 ratio in the FF, or because of blood contamination, five FF samples (5.8% of all FF samples) were excluded from any further analysis. In all analyzed FF samples, the average (±S.E.M.) E/P4 ratio was 15.6 ± 2.7.

The profiles of the concentrations of glucose, β-OHB, urea, TP, TG, NEFA and TC in serum and in the FF throughout the experimental period are shown in Figs. 2–8. The *P*-values for the effect of compartment, time and interactions compartment × time are reported in Table 1. Concentrations of TP and TC in serum transiently decreased at parturition. Glucose concentrations in serum decreased during the first 2 weeks after parturition and increased during the period thereafter. The TP concentrations stayed relatively stable after 2 weeks post partum. After a significant decrease at parturition, the serum concentration of TG remained low and TC concentrations gradually rose. Urea

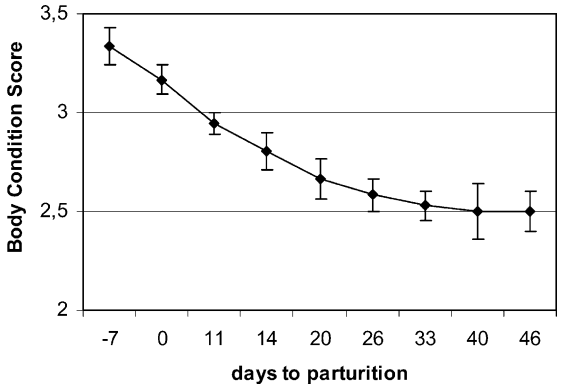

Fig. 1. Average (±S.E.M.) body condition scores of nine high-yielding dairy cows during the experimental period.

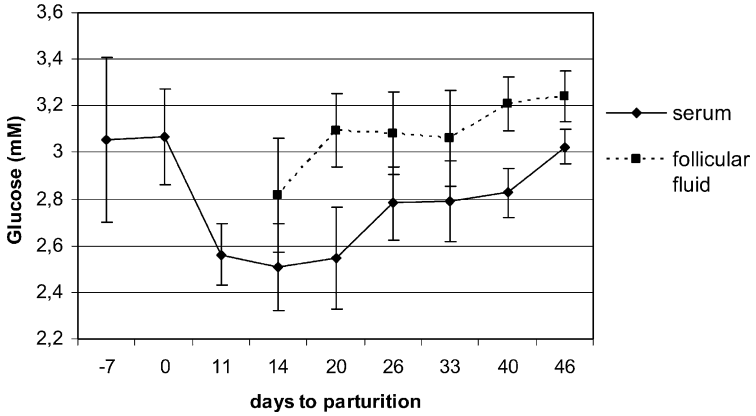

Fig. 2. Average (±S.E.M.) glucose concentrations (mM) in serum and follicular fluid of nine high-yielding dairy cows during the experimental period.

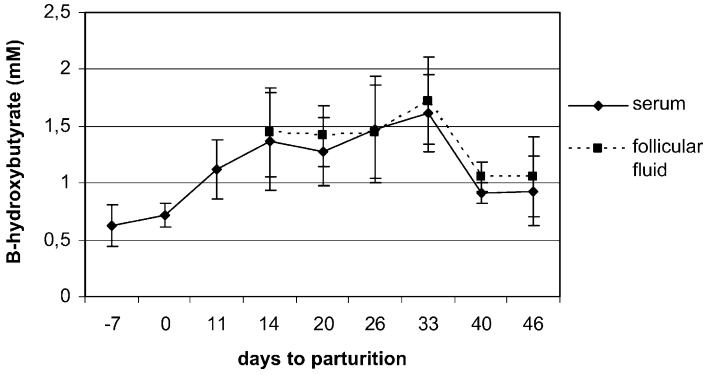

Fig. 3. Average (±S.E.M.) β-hydroxybutyrate concentrations (mM) in serum and follicular fluid of nine high-yielding dairy cows during the experimental period.

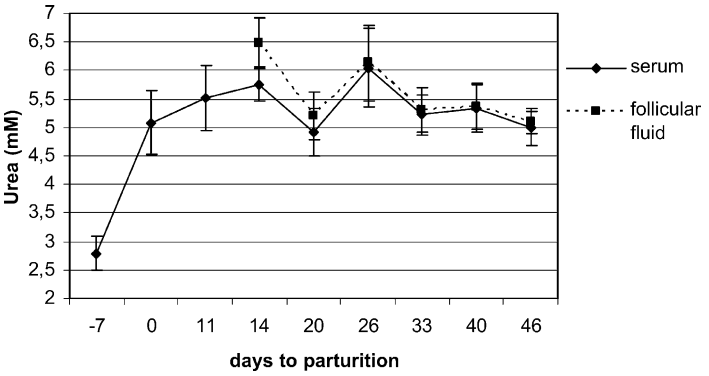

Fig. 4. Average (±S.E.M.) urea concentrations (mM) in serum and follicular fluid of nine high-yielding dairy cows during the experimental period.

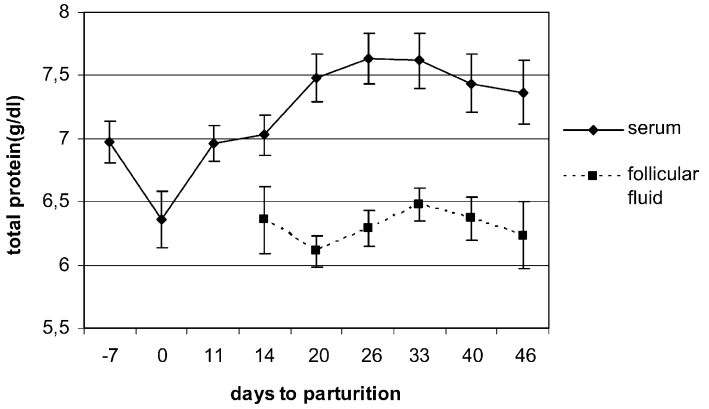

Fig. 5. Average (±S.E.M.) total protein concentrations (g/dl) in serum and follicular fluid of nine high-yielding dairy cows during the experimental period.

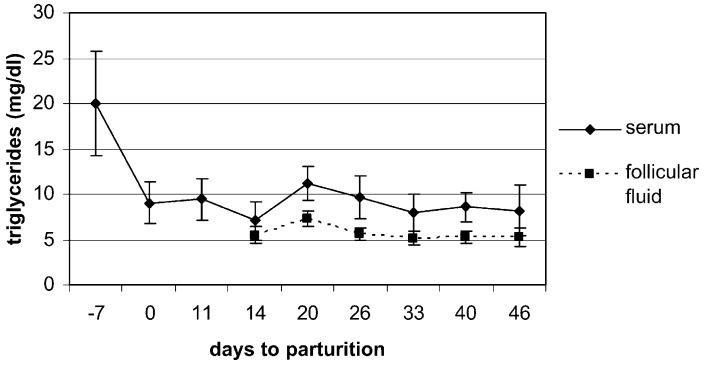

Fig. 6. Average (±S.E.M.) triglyceride concentrations (mg/dl) in serum and follicular fluid of nine high-yielding dairy cows during the experimental period.

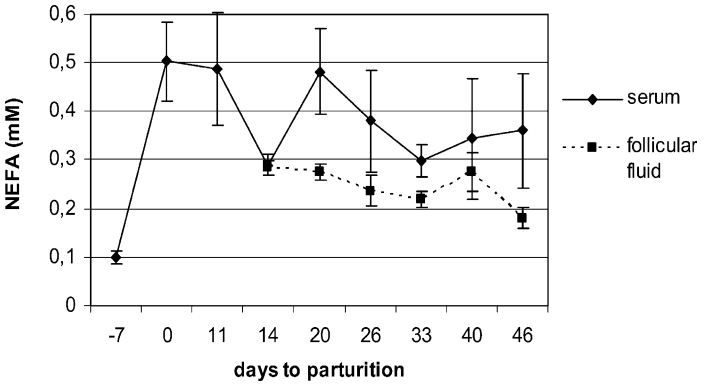

Fig. 7. Average (±S.E.M.) NEFA concentrations (mM) in serum and follicular fluid of nine high-yielding dairy cows during the experimental period.

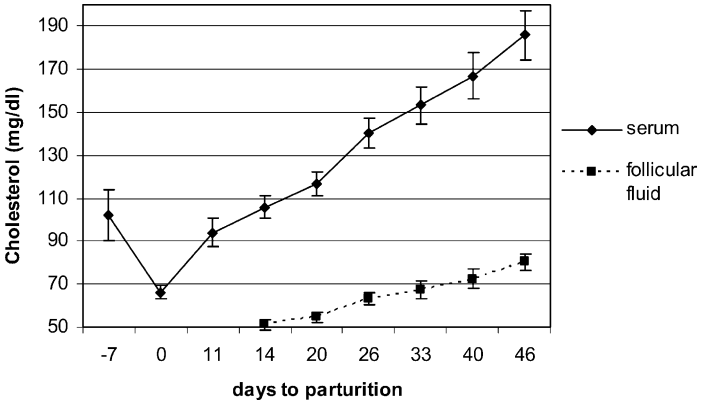

Fig. 8. Average (±S.E.M.) total cholesterol concentrations (mg/dl) in serum and follicular fluid of nine high-yielding dairy cows during the experimental period.

Table 1
Results of the linear mixed effects model (repeated measurement)

	P-values		
	Compartment effect (serum or follicular fluid)	Time effect (days post partum)	Compartment × time interaction
Glucose	**<0.01**	**<0.01**	0.40
β-OHB	0.48	**0.02**	0.91
Urea	0.25	0.06	0.56
Total protein	**<0.01**	0.60	0.73
Triglycerides	**<0.01**	0.80	0.15
log(NEFA)	**0.02**	0.05	0.86
log(cholesterol)	**<0.01**	**<0.01**	0.02

Significances of the effect of compartment, time and of the interaction compartment × time on the concentrations of the measured parameters (S-Plus, *P*-values). Bold values indicate significant effects ($P < 0.05$).

Table 2
Correlation coefficients (r's) between metabolite concentrations in follicular fluid and serum per experimental session in nine dairy cows

	Correlations (r)						
	Glucose	β-OHB	Urea	Total protein	Triglycerides	NEFA	Total cholesterol
14 days post partum	0.834*	0.996**	NS	NS	0.892**	NS	NS
20 days post partum	0.788*	0.972**	0.929**	NS	NS	NS	0.787*
26 days post partum	0.733*	0.992**	0.987**	NS	0.872**	NS	NS
33 days post partum	0.925**	0.976**	0.990**	NS	0.710*	0.845**	0.918**
40 days post partum	0.916**	0.971**	0.973**	0.860**	NS	NS	0.862**
46 days post partum	0.901*	1.00**	0.782*	NS	NS	0.908*	0.948*

Values are presented for significant correlations ($^{*}P < 0.05$; $^{**}P < 0.01$; NS: not significant).

concentrations in serum doubled around parturition and remained relatively stable for the rest of the experimental period. Serum β-OHB concentrations gradually increased after parturition and peaked at Day 33 (1.62 ± 0.34 mM). Besides a marked increase up to 0.50 ± 0.08 mM in the serum concentration of NEFA around parturition, there was no significant change in the profile during the period thereafter ($P = 0.05$).

Throughout the study the concentration of glucose in the FF was circa 0.34 mM higher than in serum. The opposite relation was found for TP, TG, NEFA and TC (Table 1, P-values of compartment effect).

Because none of the calculated interactions (compartment × time) were significant, changes in the FF for glucose, β-OHB, urea, TP, TG and NEFA during the study period were similar as changes of the same metabolite in serum (same slopes of profiles) (Table 1). For TC, however, there was a significant compartment × time interaction (different slopes of profiles) (Table 1, Fig. 8). Correlations between serum and FF levels were also calculated per experimental session post partum (without taking any time effect into consideration) and correlation coefficients are shown in Table 2. A good correlation existed at almost all experimental sessions for glucose, β-OHB, urea and TC.

4. Discussion

Characteristics of the intrafollicular environment in which the preovulatory oocyte grows and matures, may be one of the major factors determining subsequent fertility. To our knowledge, this is the first time that biochemical serum changes, are compared to changes in the FF in high-yielding dairy cows early post partum. However, FF was only sampled from Day 14 post partum onwards because of reduced approachability of the ovaries in the puerperium.

The concentration of glucose in serum showed a marked decrease during the first 2 weeks of lactation followed by a steady increase in the period thereafter. Butler [5] and others described a decrease in serum glucose concentration during the period of negative energy balance but other studies could not confirm this finding [31,32]. Landau et al. [29] showed that a low intrafollicular glucose concentration coincides with a low insulin

concentration in the FF and that the levels of both parameters are influenced by the diet. We found that the FF glucose concentration was closely correlated with the serum levels and that it was consistently higher than in serum, possibly due to an active inward transport. This finding strongly suggest that post partum changes in glycemia are well reflected in the FF of dominant follicles but that the oocyte is more or less protected from low glucose concentrations.

Glucose and β-OHB concentrations were negatively correlated, both in serum and in FF ($r = -0.56$ and -0.83, respectively) suggesting that β-OHB is a good indicator for hypoglycemia. The average serum β-OHB concentration peaked at 33 days post partum (1.62 mM). This concentration has been associated with signs of subclinical ketosis [33]. The β-OHB concentrations in serum and in the FF were similar and both slopes of profile were exactly the same. Based on these strong correlations between serum and FF concentrations throughout this study, it can be stated that elevated β-OHB levels in serum (ketonemia) will appear in the FF as well. These findings confirm what has been assumed in earlier work [24].

Urea concentrations showed an important increase during the first week post partum and remained high in the weeks thereafter. Collins et al. [34] as well as our own group [24] found in respectively mares and cows post mortem a very high correlation for urea between FF and blood serum during the first weeks of lactation. Although reports about the effect of elevated urea levels on fertility are contradictory, most authors agree that the possible adverse effect of diet induced elevated urea levels must act at the level of the oocyte [17,23,35]. Based on our results, it can be stated that elevated serum urea levels are reflected in the FF and hence, may affect oocyte quality.

The TP content in the FF remained stable during the experimental period and was about 80% of that present in serum. The similar slopes of the TP profiles in serum and FF during the study indicate that a substantial part of the protein content in FF originates from serum [27,36].

During a period of NEB, lipolysis causes an increase of NEFA concentrations in serum during the first weeks post partum. The serum NEFA levels in our study remained relatively high during the experimental period. The repeated measurement analysis of our data revealed that the NEFA concentrations in FF paralleled those in serum. This finding has been confirmed by previous studies on cows subjected to an acute dietary restriction to mimic a period of NEB [37,38]. However, the FF concentrations remained consistently lower than the levels in serum. Furthermore, there was a much higher variation in serum NEFA concentrations between animals compared to FF concentrations (an average coefficient of variation of 58 and 30%, respectively). Both findings suggest that there might be a mechanism to protect the oocyte and the granulosa cells from high NEFA concentrations, which are shown to be toxic in vitro [19,39,40].

Similar profiles of TG and TC in serum around parturition and during the first weeks post partum were described earlier and are characteristic for dairy cows [41–43]. However, the changes of TG and TC in FF during this period have never been investigated before. Following parturition, the TG concentration remained relatively low in serum and FF while the TC concentration doubled. This observation is partially caused by the mammary conversion of TG-rich lipoproteins (β-lipoproteins or very low and low-density lipoproteins, LDLs) to TC-rich lipoproteins (β-lipoproteins or high-density lipoproteins, HDLs)

[42–44]. Wehrman et al. [45] demonstrated that the TG concentration in the FF is relatively stable, regardless of an increase in the serum level due to physiological status or diet. However, dietary fat supplementation is known to increase serum and FF cholesterol concentrations [45,46]. We also found that FF total cholesterol levels rise when serum concentrations increase but both increases of TC were at different rates (75.5% in serum versus 57.3% in FF) (Fig. 8). This is confirmed by the significant compartment × time interaction we found for log(TC) (Table 1). We also found that the FF cholesterol concentration is only 43–48% (at Day 14 and Day 46 post partum, respectively) of the serum concentration. This finding suggests that the relative importance of the smallest HDL complexes (small HDL), decreased during ongoing lactation. This small HDL is the only lipoprotein fraction that can pass the blood–follicle barrier and hence is the only lipoprotein present in the FF [45,47,48]. However, the relative importance of the complete HDL cholesterol fraction (large, medium and small HDL complexes) in the amount of TC in serum remained stable during the lactation (85%) (results not shown).

In conclusion, we found that the typical post partum biochemical changes in the serum concentration of glucose, β-OHB, urea, TP, TG, NEFA and TC are well reflected in the follicular fluid of the dominant follicle. However, the oocyte and the granulosa cells seem to be protected from low glucose levels and from high NEFA concentrations.

These findings may be crucial in the understanding of the pathogenesis of sub-fertility in high-yielding dairy cattle, by affecting the quality of both the oocyte and the granulosa cells. This knowledge should be taken into account in planning further in vivo and in vitro research concerning fertility problems in high-producing dairy cattle.

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