Szent István University

Faculty of Agricultural and Environmental Sciences

Study of relation between some metabolic and reproduction parameters by laboratory methods in dairy cows

Thesis of Ph.D. dissertation

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Gödöllő
2009

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Introduction

Genetic selection of domestic animals aiming for the highest production as possible lead to altered, in serious cases to malfunction of metabolism. Metabolic diseases and, their consequences, reproduction disorders cause severe economical losses. Global efforts to increase milk production in dairy cows have been associated with a decline in fertility. Increased milk yields are not likely to be the only cause involved of this decline in reproductive performance. Probably it is the cumulated effect of non-sufficient supplementation of nutrition- and energy demand of the elevated milk production. Non-sufficient feeding of dairy cows may lead to sever physiological changes in the function of the uterus and/or the ovaries.
Nutritional background of metabolic diseases is well known but it's effect on reproduction is to be studied in depth, since the impact on profitability may not be questioned.

Objectives:

- Our aim was to survey the rate of embryonic and fetal losses in 25th and 60th days of pregnancy.
- Further aim of our study was to find metabolic blood parameters that are typical for metabolic disorders and show statistical correlations with the results of early pregnancy detection and/or with the serum progesterone levels representing ovarian activity.
- Our final aim was to find out what metabolic malfunction and nutritional effects stand in the background of reproduction disorders.

Material and methods

All experiments were carried out in Hungarian dairy farms.

Survey of the rate of embryonic and fetal losses in dairy herds

We evaluated the effectiveness of 1969 inseminations by BioPryn™ test (BioTracking LLC, Moscow, ID, USA) between the 30th and 36th days after AI on four Hungarian dairy farms.

Determination of late embryonic and fetal losses:

According to the results of our previous studies (TÓTH et al., 2005; GÁBOR et al., 2007) the BioPryn test was found an appropriate methods to determine the part of late embryonic losses between the 25th and 35th days after AI. During the optical density (OD) reading of the blood serum samples we assumed embryonic loss, if the OD of samples exceeded the cutoff OD by 10%. We assayed P4 concentration of the samples (QuantiCheck, Veterinorg Kft, Budapest). According to serum P4 concentration, cows were assigned to 3 categories:

1. pressumed embryonic loss, if P4 concentration was less than 2 ng/ml
2. possible loss, if P4 concentration was between 2-4 ng/ml
3. and maitenance of pregnancy, if the P4 concentration was more than 4 ng/ml

Rectal palpation at 60 day after AI was used to determine the fetal losses between the 36th and 60th days after AI and to check the late embryonic losses.

Effect of different handling and storing procedures on the possibility to analyze of certain biochemical parameters in plasma

The purpose of our methodological study was to determine, if the blood samples used for the pregnancy testing are sufficient for further biochemical tests.

For pregnancy detection the blood samples arrive 1 day after sampling at laboratory without separation. In the first part of our study we examined the effect of handling and storing procedures, and in the second part of the study we examined the effect of the length of freezing on the reliability to analyze different blood parameters. Blood samples were taken from the vena coccygealis and were collected in anticoagulant-containing tubes (EDTA) and fluorid containing tubes for glucose determination (S-Monovette®, Sarstedt AG & Co., Germany).

In the first part of the study blood samples were collected from 10 cows between 40-100 days after calving. Number of lactation was between 2 and 4, from the 10 animals 6 were inseminated between 60-80 days after calving. We determined the plasma concentration of glucose, total protein, calcium, potassium, urea, lactic acid dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity by reagents kits (Diagnosticum Zrt, Budapest) and the total carotene concentration using spectrophotometric methods.

The handling and storing procedures of blood samples were:

1. Separation of plasma after the blood sampling and measuring parameters immediately.
2. Separated plasma was frozen for 7 days at -18°C.
3. Separated plasma was stored for 24 hours at + 4°C.
4. Separated plasma was stored for 24 hours at +4°C, after that it was frozen at -18°C for 7 days.
5. Separating the plasma after storing the whole blood samples for 24 hours at +4°C.

6. Separating the plasma after cooling (for 24 hours at +4°C), and than freezing (at -18°C for 7 days) the plasma.

In the second part of study blood samples were collected from 8 cows between 50-110 days after calving. Number of lactation was between 1 and 3, from the 8 animals 6 were inseminated between 60-80 days after calving. We determined the plasma concentration of glucose, total protein, cholesterol, triglyceride and urea by reagents kits (Diagnosticum Zrt, Budapest) using spectrophotometric methods.

The storing procedures were:

1. Immediate measuring from plasma without storage
2. Freezing the plasma at -18°C for 7 days
3. Freezing the plasma -18°C for 14 days
4. Freezing the plasma at -18°C for 21 days
5. Freezing the plasma at -18°C for 28 days

Examination of differences between metabolic and nutritional status of pregnant and non-pregnant dairy cows

Holstein Fresian cows (n=79, average lactation number was 3) were investigated between the day 90th and 120th after calving. The average milk production was 37 kg. Two blood samples were taken from each cow in anticoagulant-containing (EDTA) and native blood collection tubes. We determined the pregnancy by BioPryn test from blood serum. The ovarian activity of non-pregnant cows was examined by P4 testing and ultrasonic examinations. During the P4 testing the cutoff of cycling ovarian activity was 2 ng/ml. In animals which had lower concentration than this cutoff we assumed lack of corpus luteum (CL) or inactive CL. Based on early pregnancy check, progesterone tested and ultrasonic examinated cows were divided into 4 groups: repeat breeder (RB), pregnant (P), open cycling (OC) and open, inactive corpus luteum (OI). To monitor the metabolic and nutritional status the samples were assayed for level of plasma α-tocopherol, retinol and β-

carotene by HPLC method, plasma concentrations of glucose, urea, uric acid (Diagnosticum Zrt., Budapest) and the plasma concentration of non-esterified fatty acid (NEFA) and β-hydroxybutyrate (BHB) (Randox Laboratories Ltd., Ardmore, UK) were assayed by spectrophotometric method. Feed samples were analyzed for DM, ash, crude protein and crude fat according to Hungarian National Standard methods (Hungarian Feed Codex, 2004). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed by Robertson and Van Soest method (1981).

The effect of antioxidant, yeast and omega-3 fatty acid supplement on metabolic status and reproductive performance in postpartum Holstein-Friesian dairy cows

All cows (n=66) received the same total mixed ration (TMR) during the dry period. Three weeks before calving feed was supplemented with extra beta carotene, vitamin E, inactivated yeast, selenium and micro elements (Supplement I.). The first 50 days post partum forage was supplemented by Glucofort 50™ (Supplement II.; Vitafort Corp., Dabas, Hungary). On fiftieth day post partum animals were divided into two groups: the control (CTR n=16) and the experimental group (EXP n=50). Feed of EXP was supplemented by 200g crunched linseed, 200 mg β-carotene, 1g yeast with selenium (2000 mg Se), and 50g yeast in 1kg/cow/day dose. (Supplement III.). The ration of CTR was not supplemented after this point of time. Feed samples (contain I., II., III: supplementation) were analyzed for DM, ash, crude protein and crude fat according to Hungarian National Standard methods (Hungarian Feed Codex, 2004). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed by Robertson and Van Soest method (1981).

To examine the metabolic and nutritional status of cows biochemical parameters were measured from the plasma in both groups (table 1).

Parameters	Date of sampling (days post partum)							
	1.	7.	15.	30.	50.	70	90.	120.
NEFA				+	+	+		
BHB	+	+	+	+	+			
Urea	+	+	+	+	+	+	+	+
FRAP	+	+	+	+	+	+	+	+
Total carotene		+		+		+	+	+

Table 1: The examined blood parameters from calving to the 120th day post partum

The cows were synchronized by Provsynch protocol and were inseminated between 70-76 days after calving. Pregnancy was diagnosed by BioPryn™ test on 30-36 days after insemination and rechecked by rectal palpation on the 60th day. Milk samples were taken three times a week before morning milking in the period: 4 day before AI to 40 days post insemination to identify the first luteal phase. P4 concentration was measured as described by NAGY et al (1998).

In this study we used digital images to evaluate the BCS of a group of cows. Pictures of cows were made when the blood samples were taken on 1st, 7th, 15th, 30th, 50th, 70th, 90th and 120th days after calving. Images were recorded for each cow, which included anterior (showed ID of cow) and posterior views (from the rear of the cow). Images were copied to a server and an independent person was asked to assess BCS based on the images. During the assessment of BCS the base score was 50 (at calving) and the decrease or increase of the condition was evaluated by +/-5 point.

Results

Survey of the rate of embryonic and fetal losses in dairy herds

According to our results in 759 pregnancy tests the ratio of embryonic and fetal losses between the 25th and 60th day after insemination was higher than 15% (n=118). The late embryonic loss was 3,29% of the total loss was detected between the 25th and 35th post insemination day by the early pregnancy detection. Majority of the total loss occurs, as fetal loss between the 35th and 60th days. We assumed 60 embryonic losses according to the result of BioPryn test, but 25 losses turned to be true. The probability of embryonic loss at >4ng/ml progesterone serum concentration was only 9%, but at <2ng/ml the probability was 86%, the observation proves the positive effect of progesterone on pregnancy retention and embryonic development. Our investigations also showed close positive correlation between milk production and embryonic and fetal losses ($r=0,93$; $p<0,001$).

Effect of different handling and storing procedures on the possibility to analyze of certain biochemical parameters in plasma

In the first part of the study we found that the concentration of glucose, calcium, potassium and urea did not change in plasma when the measuring was done from frozen samples (2). The accuracy of assay was significantly lower ($p<0,05$) when concentration of potassium, calcium and urea was measured from plasma samples that were stored at 24 hours at +4°C (3). The concentration of urea decreased significantly ($p<0,005$), when the plasma samples were stored at 24 hours at +4°C, and after that were frozen for 7 days until assay (4). Effect of storing without separation (5, 6) decreased the concentration of

glucose, potassium, calcium, urea and total protein in plasma. Effect of freezing after the plasma separation (6) increased the concentration of calcium in plasma. For the test of liver enzymes' activity only such plasma samples can be uses that were obtained by centrifugation of samples immediately after sampling (1) and the separated plasma samples were frozen until the assay. Other storing and handling methods were negative effect for reliability of the assay. The concentration of total carotene did not change for the effect of different handling and storing procedures.
In the second part of the methodological study it showed that 4-week-long freezing did not affect the reliability of measuring glucose, cholesterol, urea and triglyceride. The blood plasma of total protein concentration increased in the 4th week of freezing.

Examination of differences between metabolic and nutritional status of pregnant and non-pregnant dairy cows

We examined the differences between the concentrations of biochemical blood parameters in cows, grouped by reproduction status.
The concentration of α-tocoferol, β-carotene was higher in the samples of repeat breeders (RB) and the open, inactive CL (OI) groups, than the pregnant (P) and the open cycling (OC) groups. We found significant differences ($p<0,05$) between the NEFA concentration of groups, we measured low concentration in OC and P groups. The concentration of NEFA did not exceed the physiological value in the group of P. Elevated NEFA concentration was detected in 8,3% of OC, in 20% of OI and in 31,6% of RB groups. The BHB concentration was significantly lower ($p<0,05$) in RB cows than the P cows. The concentration of uric acid was elevated in OC (4/24), in RB (2/19) and P (4/21) groups, but the concentrations were physiological. The concentration of uric acid was significantly lower in OI (38,9 μ

mol/l) group than in the P (46,5 μmol/l) group. The urea concentration was normal in both groups. The average concentration of β-carotene was lower than the physiological range (3000 μg/l). We find extremely low concentration of β-carotene in 83,3% of OC, in 64,7% of OI, in 66,6% of P and in 73,3% of RB. There were not significant differences between the glucose and retinol concentration of groups in plasma.
Significant correlation ($r=0,69$, $p<0,001$) was found between the concentration of α-tocoferol and β-carotene. Positive mild correlations were found between the plasma level of retinol and urea ($r=0,33$, $p<0,01$); uric acid and β-carotene ($r=0,21$, $p<0,05$); and BHB and urea ($r=0,3$, $p<0,01$). Negative correlation was observed between the concentration of β-carotene and urea ($r=-0,33$, $p<0,01$); β-carotene and BHB ($r=-0,28$, $p<0,05$); and urea and glucose ($r=-0,20$, $p<0,05$).

The effect of antioxidant, yeast and omega-3 fatty acid supplement on metabolic status and reproductive performance in postpartum Holstein-Friesian dairy cows

To increase energy intake of cows their feed were supplemented by Glukofort™ (from calving to 50. days) and omega-3 fatty acid, antioxidant, and yeast containing supplement (from 50. days after calving). Body condition of cows decreased from calving to the 50th days, but improved in parallel with the decreasing of milk production. The omega-3 fatty acid supplement had positive effect on milk production; the milk production of experimental group was higher 3,6kg/cow/day than the control group. Although, we did not find significant differences between the body conditions of groups; the loss of condition was more distinct after calving inspite of the lower milk production. The nadir of body condition in control cows was on the 50th day after calving, when the milk production also was decreased. The concentration of NEFA was elevated on the 30th and the 50th

days after calving, but this concentration on 70th was normal and did not differ between the groups. The concentration of BHB did not exceed the normal value in the experimental period, when the feed was supplanted by Glukofort. The concentration of urea was physiological and the concentration was significantly lower in the experimental group on 90th and 120th days post partum. We used FRAP concentration to evaluate the antioxidant-capacity of plasma. The concentration of FRAP was physiological in the experimental period and was no differences between the groups. The carotene supplementation from the 50th day after calving resulted in significantly higher total carotene level in the blood samples.
The fertility results of the experimental group (pregnancy rate for the first AI 41,2%) was lower than in the control group (57,1%). In the supplemented cows' samples the milk progesterone level after insemination was higher than in the control samples. Greater ratio of the experimental group (more than 80% of cows) showed cyclic ovarian activity than in the control group (65% of cows). In our study the pregnant cows in the experiment group had higher progesterone concentration between the 15th and 39th days of pregnancy than the control cows.

New scientific results

1. We determined that the rate of embryonic and fetal losses are 15,5% between 25-60 days after insemination.
2. We defined that the concentration of potassium, calcium, glucose, triglyceride, cholesterol and total protein does not exceed the physiological values between 40-110 days after calving, which means that their concentration does not refer the negative energy status of cows in this period.
3. Based on our results we concluded that, due to the sufficient energy supply between the 90th-120th days after calving there is no significant correlations among the conception, the ovarian activity of open cows and the concentration of βOH-butyrate, the non-esterified fatty acids and urea which means that in the first month of pregnancy cows compensated the negative energy balance occurring at the beginning of the lactation.
4. In pregnant cows fed by high content of omega-3 fatty acids, yeast, β–carotene and selenium supplement their milk-progesterone concentration increased significantly between the 15^{th} and 39^{th} days after insemination which supports the pregnancy maintenance. The higher level and faster post ovulation increase of progesterone has positive effect on the early embryo development and decreases the possibility of early embryonic losses.
5. The feed with high content of omega-3 fatty acids, yeast, β–carotene and selenium supplement had positive effect on the cows' cycling ovarian activity and progesterone secretion after the 50^{th} day after calving.

Conclusions and recommendations

Survey of the rate of embryonic and fetal losses in dairy herds

In our survey the rate of embryonic and fetal losses between the 25th and 60th days after insemination was higher than 15%. This finding agreed with the study of VASCONCELOS et al. (1997), who find that embryonic and fetal loss rate higher than 20% in high production dairy cows. Our investigations also showed that the increase in milk production was in positive correlation with the embryonic and fetal losses. In cows with >45kg/day milk production the losses were 30% which drives the attention to the possible negative effect of one-way selection and the increased nutritional demand of the high potential herds.

Effect of different handling and storing procedures on the possibility to analyze of certain biochemical parameters in plasma

Based on our experience we found that from blood samples that were not separated immediately after sampling detection of glucose, potassium, calcium, urea and total protein concentration, ALT, AST and LDH is not reliable. Based on this observation it can be concluded that until the centrifugation of the blood samples in order to separate the plasma or the serum further biochemical reactions take part that have negative influence on the reliability of the tests (except total carotene). Based on the above experience it can be concluded that for the test of liver enzymes' activity only such plasma or serum samples can be use that were obtained by centrifugation of samples immediately after sampling. Separated samples can be stored at +4ºC but not longer than for 24 hours. Otherwise separated samples should be stored frozen (-18 or -70ºC) until analyses. As a summary of this methodological study it can be

concluded that whole samples used for pregnancy testing can not be used for those laboratory measuring of the blood parameters chosen by us.
Between the 40th and the 110th days after calving the concentration of calcium and potassium, that were chosen to measure nutrition status, the concentration of glucose, total protein, triglyceride and cholesterol that were chosen to measure fatty acid, carbohydrate and protein metabolism did not exceed the physiological range. Based on this observation it can be concluded that the above mentioned parameters are not relevant to track nutritional or metabolic status.

Examination of differences between metabolic and nutritional status of pregnant and non pregnant dairy cows

We find statistical differences between the blood sample of cows with different reproduction status and ovarian function and their nutritional and metabolic status. Although, these differences were statistically significant were not physiologically important. Based on our results we concluded that, due to the sufficient energy supply that by the 90th-120th day after calving cows compensated the negative energy balance occurring at the beginning of the lactation.

The effect of antioxidant, yeast and omega-3 fatty acid supplement on metabolic status and reproductive performance in postpartum Holstein-Friesian dairy cows

In accordance with results of PETIT et al. (2001; 2002; 2004) as the effect of omega-3 fatty acid supplementation milk production was higher. The concentration of BHB did not exceed the normal value in the experimental period, which means that glucogenetic supplementation had positive effect on energy balance of cows. In several studies (MOALLEM et al, 1997; DRACKLEY et al, 1992; OLDICK et al, 1997) the fat supplementation lead elevated NEFA concentration in plasma.

In our study the concentration of NEFA exceed the physiological value on 30th and 50th days after calving but by the 70th day decreased to the normal range. We concluded that the applied supplements had positive effect on energy status of cows. In the supplemented cows' samples the milk progesterone level after insemination was higher and increased faster than in the control samples. Greater ratio of the experimental group showed cyclic ovarian activity which means that the omega-3 fatty acid, yeast, β-carotene supplementation had positive effect on ovarian function. The supplementation did not correlate with the fertility rate but had positive effect on the progesterone level of pregnant cows. It could also be concluded that the higher progesterone concentration detected between the 15th and 39th days of pregnancy decreases the probability of embryonic and fetal losses (DARWASH and LAMMING, 1998).

Our results indicate that by assembling the optimal poly-unsaturated fatty acids content in the feed we can influence the P4 production and the maternal recognition of pregnancy, which decrease embryonic losses.

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