SZENT ISTVÁN UNIVERSITY
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NEW DATA TO THE PATHOPHYSIOLOGY, CLINICS AND THERAPY OF BOVINE MASTITIS

THESIS of the PhD DISSERTATION

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GENERAL INTRODUCTION

Bovine mastitis can be defined as the response of the udder against various stimuli, particularly invading microbes. Acute mastitis is characterized by the classical signs of inflammation, such as swelling, pain, tenderness of the udder, fever and loss of function. The cow often fails to clear up the infection: mastitis pathogen bacteria persist for months within the udder and are shed in the milk. Chronic inflammation results in decreased milk production with increased milk somatic cell count.

Bovine mastitis has a great impact on the economy of dairy industry. The disease causes decrease in milk production and in milk quality resulting in severe losses for both the producers and processors of milk. An additional loss is represented by the cost of antimicrobial therapy and the discarded milk contaminated with drugs.

As a result of the changes of feeding, housing and milking systems and by the development of different mastitis control programs the epidemiology of this disease has dramatically changed. This process is noticeable also in Hungary.

Dairy farming has changed dramatically in the last century. In 19th century the average number of cows per farm was below five and the animals grazed in fields in the summer, and they were kept in small barns in winter on soil straw litter. The cows were fed mainly dry hay and milked by hand. The average annual milk production was about 2000 kg per cow. The prevalence of mastitis at that time is not known, but seemingly it was a minor problem that time. Since the beginning of the 20th century a concentration of farmed cows started in Europe and also in Hungary. This process was associated with more intense feeding and keeping which led to higher milk production but unfortunately it came with a higher risk of production diseases including mastitis too. Several studies demonstrated that high-producing cows are at increased risk of infectious diseases.

The mastitis caused by Strep. agalactiae was the first case of mastitis resulting in high economic losses in Hungarian dairy farms.

After introducing antibiotics into the mastitis therapy (1945), most of the highly susceptible Strep. agalactiae strains had been replaced by bacteria such as *S. aureus* which are more resistant to the antibiotic therapy in the udder. At present time Staph aureus is the most frequent contagious mastitis-pathogen germ in Hungary (unpublished data of the Central Veterinary Institute, Budapest). The milking machine is the main source of new animal to animal intramammary infections by *S. aureus*. In addition, the improper machine milking is an important factor to support bacteria to colonize teat ends and reduce the capacity of self defense mechanism of the udder

Despite of efforts of several researchers worldwide there is no antibiotic and treatment regime, which is able to eliminate *S. aureus* from the udder and reliably cure the chronic udder inflammation.

In the past decade, the standard mastitis control program has suggested hygiene and management practices to control intramammary infection. A decrease in bulk milk somatic cell counts (SCC) is an indicator of the success of the control programme. Although farmers with low SCC herds were able to decrease the prevalence of mastitis with contagious pathogens, these herds still show a high incidence of clinical mastitis by environmental pathogens. In several low SCC herds coliform bacteria (mainly E.coli) are major causes of clinical mastitis.

Low somatic cell count in milk seems to predispose to coliform mastitis. Somatic cells are parts of the defense mechanism of the udder. The udder becomes more sensitive to coliforms as the SCC decreases.

An increased incidence of clinical mastitis caused by environmental pathogens was reported to be directly associated with impairment of cow defence mechanisms mostly in the early postpartum period.

Transition from pregnancy to lactation involves considerable metabolic adaptations in all mammals especially in cows selected for a high rate of milk production due to the sudden demand for large quantities of glucose in an animal with a ruminal digestive system. Rapid adaptation requires immediate changes in the rates of synthesis-secretion and degradation-elimination of endocrine regulatory molecules which is reflected by characteristic changes in their circulating concentrations. However, the ability of a cow to adapt successfully may be compromised during episodes of postparturient mastitis and other infections, particularly those caused by Gram-negative pathogens, which release lipopolysaccharide (LPS) from the outer cell wall membrane during the inflammatory process.
The pathogenesis of coliform mastitis is based on the host response to endotoxin originated from the cell wall of bacteria. During this process several inflammatory mediators (tumor necrosis factor, interleukines etc.) release from leukocytes. These mediators can cause both severe inflammatory symptoms in the udder and disorders in the metabolic and endocrine functions of the cow.
The present Hungarian dairy herds are on different stages of this evolution of mastitis epidemiology.
In my Ph.D. work I studied some special aspects of bovine mastitis having practical importance in Hungarian dairy herds.

AIMS OF THE STUDY WERE TO

1. Evaluate whether the predisposition for clinical mastitis resulted from contagious or environmental pathogens in the first some weeks of lactation is influenced by the immediate postpartum metabolic condition of the cow.
2. Evaluate the known periparturient tendencies in plasma levels of certain metabolic hormones influenced by mastitis in puerperium.
3. Evaluate whether the endocrine alterations known from model studies can be recognized in pathogenesis of clinical mastitis also in the practice
4. Evaluate whether the presence of temporary hypocorticism interferes with the course of this disease.
5. Evaluate the efficacy of intramuscular vs. intracisternal spiramycin dry cow therapy against *S. aureus* mastitis.
6. Evaluate the microbiology, pathology and epidemiology of the bovine mastitis caused by the alga Prototheca zopfii

MATERIALS AND METHODS

Chapter 1: METABOLIC AND ENDOCRINE ASPECTS OF BOVINE MASTITIS IN EARLY WEEKS OF LACTATION

Design. Farm conditions. Experimental animals

The trial was carried out in 4 commercial large-scale dairy units with about 500 to 1850 Holstein-Friesian cows and their crosses in each, producing about 7000 - 8300 kg fat corrected milk per cow. Each farm had its own mastitis control program (Herd A represented one of the top farms of Hungary), and was free of *Streptococcus agalactiae.* All 4 farms could produce low somatic cell count milk (LSCCM) for many years. In order to avoid the age-related interference only ≥2 parity cows were involved in the trial. The course of calving was normal in all of them. The systemic administration of glucocorticoids and non-steroidal antiinflammatory agents were not allowed in this study, because we wanted to avoid their interference with the endocrine consequences of mastitis.

Exp. 1 **(in Herd A only)**

Fifteen cows were selected for this trial some days before their expected delivery. No clinical symptoms of any diseases had been observed in these cows during the previous 60-day dry period. Blood samples for endocrine and metabolite assays were taken from them at 8.00 h AM on days 4-5 and 2 prepartum and on days 1-2 and 3-5 postpartum, as well as at the onset of delivery on the day of parturition.

If outbreak of mastitis was observed *simultaneously with calving or in the first 5 days postpartum*, a standard procedure consisting of clinical examination, milk sampling for bacteriology and therapy was carried out. At the end the data of cows affected vs. not affected by various forms of mastitis were compared.

Exp. 2 **(in all of the four herds)**

All of cows which (1) were free from any clinical symptoms of chronic mastitis, and (2) calved within the pre-selected periods of the study were involved in the trial, unless they needed veterinary intervention at calving, calved twins, and/or showed clinical symptoms of parturient paresis, hepatic injuries or mastitis before taking blood samples on d 1-3 after calving.

During these pre-selected study periods the farms were visited twice a week and all the cows (n = 335) calved 1 to 3 days before and met the above requirements were enrolled in the study. 60 to 90 min. after the morning milking (and before the morning feeding) a blood sample was taken from each of them for determination of certain hormones, metabolites and enzymes known to be related with the energy metabolism and liver function. Also the adrenocorticotrop hormone (ACTH) challenged cortisol release and the thyreotrop-releasing hormone (TRH) induced T_4 and T_3 responses were determined.

If after this day 1-3 sampling procedure mastitis was diagnosed *in the first 28 days* of lactation, the standard clinical examination, milk sampling for bacteriology and treatment were performed. At the final evaluation data of non-mastitic cows were compared to those with mastitis caused by groups of the various pathogens. The course of uterine involution was checked by rectal palpation and vaginoscopy in all cows on day 6-14 after calving.

Exp. 3 **and** ***4*** **(in Herd A and D)**

Cows affected by mastitis in the *early* and *late puerperium*, or during their *peak lactation* (e.g. on day 0-14, 15-28 or 29-60 after calving, respectively) were enrolled in the study. In *Exp. 3* also the presence of systemic symptoms of this disease was involved in the inclusion criteria. As controls, their healthy counterparts (being almost identical in parity,

stage of lactation and current milk yield) were selected in the same herd in both of these experiments.

After diagnosing the outbreak of mastitis the standard clinical examination was performed, milk samples were taken for bacteriology and treatment was administered. In *Exp. 3* blood samples were taken for endocrine and metabolite determinations at 14.00 h, and again at further 5 subsequent times 6 h apart (e.g. at 20.00 h, 02.00 h, 8.00 h, 14.00 h and 20.00 h). All samplings were preceded by complete milking out at least 90 min. earlier. Simultaneously with this blood sampling milk samples were also taken from the non-affected quarters for assaying the progesterone (P_4) content (in cows after the colostral period only). In *Exp. 4* the ACTH challenged cortisol release and the TRH induced T_4 and T_3 responses were determined (administering the ACTH / TRH at 14.00 h). Only the challenging and sampling processes were performed, however, in the healthy counterparts.

Clinical examination. Isolation and identification of pathogens.Data evaluation

In all of the experiments as symptoms of mastitis were observed in cows involved in the trial, the affected individual was separated immediately for clinical examination, sampling for bacteriology and receiving the standard treatment procedure. The pathogen was isolated and identified.

Mathematical analyses were made by the statistical package SPSS for Windows 8.0.

The assay procedures used for determination of enzymes and metabolites

Parameter		Technique
Aspartate aminotransferase (AST)	Exp. 2	IFCC, UV method
Glucose	Exp. 2	Enzymatic (GOD-POD reaction
Acetoacetate (ACAC)	Exp. 2	Salicylaldehyde reaction
βOH-butyrate (BHB)	Exp. 1, 2 and 3	βOH-butyrate dehydrogenase reaction
Non-esterified fatty acid (NEFA)	Exp. 1, 2 and 3	Extraction of their colored soaps
Total cholesterol (TCh)	Exp. 2	Enzymatic (CHOD-PAP) reaction
Trigliceride (TG)	Exp. 2	Enzymatic method
Urea	Exp. 2	Enzymatic (urease) reaction

The endocrine assay procedures

Hormone	Technique	Trial
Hormones from defatted milk samples		
Progesterone (P_4)	Microplate ELISA	Exp. 3
Hormones from blood (heparinized plasma) samples		
Progesterone (P_4)	Direct ^{3}H-RIA	Exp. 1 and 3
Cortisol	Direct ^{3}H-RIA	Exp. 1, 2, 3 and 4
Thyroxin (T_4)	^{125}I-RIA	Exp. 1
		Exp. 2, 3 and 4
3,3',5-triiodo-thyronine (T_3)	^{125}I-RIA	Exp. 1
		Exp. 2, 3 and 4
3,3',5'-triiodo-thyronine (rT_3) [h]	^{125}I-RIA	Exp. 2 and 4
Insulin	^{125}I-RIA	Exp. 1
		Exp. 2
		Exp. 2, 3 and 4
Insulin-like growth factor-I (IGF-I)	Extraction and heterologous ^{125}I-RIA	Exp. 2 and 3
		Exp. 1, 2
Insulin-like growth factor-II (IGF-II)	Extraction and heterologous ^{125}I-RIA	Exp. 1

Chapter 2: BACTERIOLOGICAL RECOVERY OF *S. AUREUS* MASTITIS AFTER INTRAMUSCULAR OR INTRACISTERNAL SPIRAMYCIN-BASED DRYING OFF THERAPY

Herds and Animals

This study was conducted in three commercial large-scale dairy herds. Fifty-seven, 19 and 24 cows with subclinical mastitis (SCC >500,000 in the last three months of their lactation) were included in the study on farms 1, 2 and 3, respectively, but only those with *S. aureus* IMI were used for the final evaluation. Cows were dried off approximately 60 days before the calculated calving dates. During the dry period the cows were housed in separate stalls and about 1 week before the estimated calving date they were moved to the maternity unit where they stayed until postpartum day 5-7.

Sampling and Laboratory procedures

From all udder quarters of the cows aseptic milk samples were collected for bacteriological culture during the last milking out, at drying off and again in the first week after calving. The isolation and identification of pathogens were made. The SCC of milk was also determined (Fossomatic technology) for each quarter just before drying off, on postpartum days 3-4 and then once a week for further 4 weeks postpartum.

Treatments

The cows were randomly divided into four treatment groups. (1) The animals in the first group served as untreated *controls* (number of cows : n_{cow} =21) and no antimicrobials were given to them at drying off. Those in the second and third groups received an intramuscular injection of 30,000 IU/kg spiramycin base (5ml/100 kg; Suanovil^{R} 20, Rhone Mérieux) (2) in a single dose (*single IM group*; n_{cow} =25) or (3) for 4 consecutive days (*4 IM group*; n_{cow} =30). (4) An intracisternal preparation with 1.2 million IU spiramycin and 100 000 IU neomycin

bound to a long acting excipient (Speciorlac^R, Rhone Mérieux) was administered into each of the udder quarters in the last group (*intracisternal group*; n_{cow} =24). All treatments were administered immediately after the last milking out at drying off.

Chapter 3: PROTOTHECA ZOPFII MASTITIS IN HUNGARIAN LARGE SCALE DAIRY HERDS

Antecedents

In autumn of 1997, a yeast-like microorganism was cultured from some mastitic milk samples collected for an udder health control programme. This pathogen was subsequently identified as the unicellular alga of *P. zopfii*. Since then, the same organism has been isolated from more than 50 large-scale farms on at least one occasion. The accumulated outbreak of this masitis has been observed in 10 of these farms (*endemic appearance*). Three of these dairy herds with a high incidence of algal mastitis were closely studied (*farm survey)*. As a third step the epidemiological character of this infection was followed on one of the farms.

The farm survey

Each of the three farms involved in this survey had 200–300 Holstein-Friesian × Hungarian Red Spotted crossbred cows kept.

Altogether 73 mastitic cows were involved in the *farm survey*. After the clinical examination, aseptic milk samples were collected from each quarter for microbiological investigations. If the quarter proved to be infected, it was re-sampled again several times 4 to 5 weeks apart during a 12-18-months long period. Simultaneously, the SCC was also checked. The SCC data of the previous lactation were available for retrospective evaluation. If cows dried off, the sampling procedure was re-continued after calving. Also feed (maize silage, wet sugar-beet chips), faecal and bulk milk samples (only in *Herd 3*) were taken for microbiological investigations. In *Herd 3* the milk samples taken from quarters of 15 alga-infected cows were cultured also for *mycoplasmae*.

Histopathology

In the *on-farm survey phase* three alga-infected cows were slaughtered and their udders were subjected to gross and histopathological examination.

RESULTS

In **CHAPTER 1** the cows affected by mastitis in the puerperium had shown more elevated AcAc, BHB, NEFA and rT_3, and lower IGF-I, T_4 and T_3 levels previously than those remained healthy during the first 4 weeks after calving (*Exp. 1, 2*.). This tendency related to a more severe form of energy imbalance and derived mainly from parameters of mastitic cows infected with GP and GN environmental pathogens or affected by NDP mastitis. However, just after calving the data of those with *S. aureus* IMI were very close to their healthy herdmates. Significant predictive value was attributed only to the elevation of BHB, but not to any others of NEB related changes in circulating levels of hormones and metabolites. This predictive value was highly significant for GN microbes. Based on these findings we suppose that in the early weeks of lactation rather the hyperketonaemia than the NEB itself can predispose the cow for mastitis.

Elevated BHB levels were detected in the first samples of cows with NDP+GN mastitis taken within some hours after the outbreak of clinical symptoms. The BHB dependent character of NDP+GN mastitis was obvious in the first four weeks after calving. During the

sampling period the BHB levels started to decrease and reached the physiological range within some hours.

Contrary to this continuously decreasing tendency in BHB, in the first some hours of the course a temporary elevation of NEFA level was seen in cows with NDP+GN mastitis, but not in those with GP mastitis. The observed NEFA increase might be the catabolic consequence of endotoxin induced endocrine changes.

About 23 % of the cows in *Exp. 2* showed < 40.00 nmol/l (e.g. lower than the mean - SD of symptomless, normoketonaemic cows; n=199) cortisol response to the ACTH challenge and were considered as *temporary hypocorticoid*. At the time of challenge all the cows were healthy, and neither the baseline level of cortisol, nor the degree of ACTH-induced cortisol response predisposed the cow for mastitis. However, if *hypocorticoid* cows were affected by GN or NDP mastitis in the first 14 days after calving, they showed more severe clinical symptoms and had higher risk for a fatal course, than their normocorticoid counterparts.

The LPS-induced cortisol increase was also detected in the *Exp. 1, 3* and *4* of the current trial. However, in our cases: in the 3 cows died in the earliest few days after calving in *Exp. 3*. no cortisol increase was detected at all. The cows in *Exp. 4* showed significantly lower ACTH-induced cortisol response in the early puerperium, than in the later stages of lactation. The two cows died of *E. coli* and NDP mastitis could hardly response to the ACTH challenge. The clinical outbreak of mastitis was diagnosed in both of them also in the early puerperium. In cows with NDP+GN mastitis in the early puerperal phase the ACTH challenged cortisol increment inversely related to the severity of clinical symptoms.

These experiences have confirmed the regulatory role of physiological cortisol response in production and release of certain interleukins and TNFα in GN mastitis, emphasizing the clinical importance of temporary hypocorticism in postpartum dairy cows and the clinical importance of anti-inflammatory therapy in mastitic cows showing severe general symptoms of this disease.

Simultaneously with the LPS-induced cortisol elevation also a slight, temporary *progesterone* (P_4) increase was seen in cows with NDP+GN mastitis. This temporary P_4 increment was detectable only when no active corpus luteum was present on the ovary, and it was considered to be of adrenal origin as a side product of the LPS-forced cortisol synthesis.

Studying the thyroid gland function significant decrease in plasma levels of both the T_4 and T_3 (in *Exp. 3*), and diminished TRH-challenged T_4 and T_3 increase (in *Exp. 4*) were deteced, probably due to the cytokine loading must have been the most intensive in the severe forms of NDP+GN mastitis. These mastitis related changes were more obvious in more advanced stages of the course (in *Exp. 3*), were more pronounced in the early puerperium than in the late puerperium or during peak lactation (both in *Exp. 3* and *4*), and were extremely dramatic in the few cases died of mastitis soon after sampling. Contrary to the experiences in cows with NDP+GN mastitis, only mild or no mastitis-induced alterations were detected in animals affected by GP mastitis. The cows with NDP+GN mastitis were characterized by significantly more elevated rT_3 levels. These –supposedly endotoxin mediated – differences derived from the data of the most severe cases. This observation reveals that due to a mastitis related endotoxin loading in cows not only the 5'D-dependent activation of T_4 to T_3 may be impaired but also the capacity of its 5D-katalyzed inactivating pathway to rT3 can be increased: this may be a significant contribution for the LPS-induced decrease of T_4 in plasma.

The low prepartum T_4 levels in the cows which later exhibited GN mastitis may indicate either that cows with low T_4 status were more susceptible to infection by GN organisms or that the endotoxin-released products were already acting on the thyroid gland before clinical mastitis was evident. The immunosuppressive consequences of decompensated NEB (associated with low T_4 status), rather than the low plasma levels of T_4 and T_3 by themselves were supposed to predispose the cows for mastitis in *Exp. 1* and *2*.

The insulin and IGF-I levels of our cows in *Exp. 2* reflected the energy plus protein (but mainly energy) balance, and the low day 1-3 level of IGF-I in cows showing mastitis some days later confirm only the higher susceptibility of these individuals to intramammary infection caused by environmental pathogens in the early weeks of lactation. Significant independent associations of serum T_4 with T_3 and IGF-I levels during the puerperium were found in the *Exp. 1* and *2.*

A temporary elevation in plasma insulin was usually observed also in the first 2-3 samples of our cases with NDP+GN mastitis in *Exp. 3*. In complete agreement with the findings of model studies the IGF-I level was still almost unaffected at the time of this insulin elevation, and started to decrease continuously thereafter. Similar changes in insulin and IGF-I were seen almost never in cows with GP mastitis. These results have confirmed that the endotoxin-induced changes of both the GH-IGF-I axis and insulin participate in the shift of the metabolism towards catabolic events also in ruminants including the postpartum dairy cows.

Based on these results, we conclude that GN- (endotoxin-) mastitis related alterations in metabolic and endocrine systems may have practical importance in postpartum dairy cows. So it may be justified to exclude the mastitic cows (or at least of those with sever general symptoms) from trials studying the endocrine, metabolic and reproductive effects of certain feeding technologies and/or treatment procedures.

In **CHAPTER 2** systemic (intramuscular, IM) vs. local (intracisternal, IC) routes of spiramycin-based drying off therapy were compared for efficacy on 65 *Staphylococcus aureus* infected udder quarters of 38 dairy cows. Single-dose (30 000 IU/kg) IM treatment (*single IM group*) resulted in a similarly low bacteriological recovery rate (14%) as seen in the untreated controls (18%). IM treatment (30 000 IU/kg) on 4 consecutive days (*4 IM group*) resulted in significantly higher quarter-based recovery rates than that in the *single IM group*. The bacteriological recovery rates obtained in the *intracisternal* and *4 IM groups* were quite similar but remained below 50%. Based on these findings as well as on the high costs of repeated intramuscular treatment regime there is no reason to give extra preference to the systemic application of spiramycin at drying off in the practice.

In **CHAPTER 3** the bovine mastitis caused by *P. zopfii* alga is discussed. This form of mastitis have not been reported from Hungary previously. In the years 1998-99 223 in 32 large-scale dairy herds cases of bovine mastitis by *Prototheca zopfii* were identified by the author. In the following years algae were isolated from several cows in more than 50 dairy herds. The ratio of algal mastitis increased from 2 % to 4.5 % in years 1999 and 2001, respectively. All of these farms were in Hungary, at a continental type, temperate zone climate. Both the sporadic and epidemic forms of *P. zopfii* mastitis were observed. Three of these dairy herds with a high incidence of algal mastitis were closely studied (*farm survey).* All the herds affected by the epidemic form had poor hygienic conditions and suffered from several managerial faults, but no specific predisposing factors could be identified. In most of the cases the *type II* variant of this pathogen was isolated. However, from 3 cows *type III* variant of *P. zopfii* was also isolated. This variant has not been isolated previously from bovine mastitis cases.

The cows had a higher chance of new infection in the early weeks of lactation and in the summer. The *P. zopfii* infection usually resulted in a chronic subclinical, or a mild clinical, inflammatory process in the udder followed by a dramatic loss in milk production and permanent increase in somatic cell count. The histopathological findings could be characterized, as a progressive interstitial mastitis associated with alveolar atrophy. The self-recovery rate was very low.

Prototheca zopfii is concluded as a common mastitis pathogen of the dairy cows in Hungary as well.

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